



ACTA PHYSIOLOGICA SCANDINAVICA  
VOL. 69 SUPPLEMENTUM 281

FROM THE DEPARTMENT OF ANATOMY UNIVERSITY OF HELSINKI  
SILTAVUORENPENGER, HELSINKI FINLAND

HISTOCHEMICAL REACTIONS OF THE  
ENTEROCHROMAFFIN CELLS AND THE  
5 HYDROXYTRYPTAMINE CONTENT  
OF THE MAMMALIAN DUODENUM

BY  
ANTTI PENTTILÄ

HELSINKI 1966

Helsinki 1968  
Maalaiskuntien Liiton  
Kirjapaino

## PREFACE

The subject of the present study was proposed in 1964 by Professor Olavi Eranko M D Ph D Head of the Department of Anatomy University of Helsinki. This work is a part of the departmental research project on the histochemistry of biogenic amines.

I wish to express gratitude to Professor Eranko for his encouragement continued support and valuable criticism from the first experiments to the preparation of the manuscript.

Professor Matti Paasonen M D read the manuscript and suggested significant alterations for which I am very grateful to him.

I wish warmly to thank my colleagues in the Department of Anatomy who have given me valuable advice and help. My thanks are due especially to Dr Aulikki Kokko M D Docent Matti Harkonen M D Dr Arvi Kahri M D and Dr Boris Kock M L.

I also wish to thank my brother Dr Ilkka Penttilä M D for many illustrative and valuable discussions.

My thanks are further due to Mrs Lusa Kangasvuo for her assistance in the preparation of histochemical specimens and Miss Helmi Palomäki for help in obtaining the literature.

Miss Elvi Kaukokallio corrected the English.

Finally I wish to express my warmest thanks to my wife Sisko Penttilä M A for all her help and correction of the English manuscript.

Helsinki July 1966

*-Aatto Penttilä*





# CONTENTS

INTRODUCTION	7
REVIEW OF LITERATURE	8
The Enterochromaffin System	8
Enterochromaffin Cells in Different Species	9
<i>Distribution of the Enterochromaffin Cells</i>	9
Morphology of the Enterochromaffin Cells	10
Histochemical Reactions of Enterochromaffin Cells	10
Fixation of Enterochromaffin Cells	10
Fluorescence Reaction	11
Silver Reactions	12
Dialo Coupling Reaction	13
Indophenol Reaction	13
Ferric Ferricyanide Reaction	13
Ferric Chloride Reaction	13
Ninhydrin Reaction	14
Oxidative Agents	14
Other Reactions	14
Chemical Character of the Enterochromaffin Granules	15
Catechol Theory	15
Pteridine Theory	15
Resorcinol Theory	15
5 Hydroxytryptamine Theory	15
5 Hydroxytryptamine in the Gastrointestinal Tract	16
PROBLEMS OF THE PRESENT STUDY	18
MATERIAL	19
METHODS	20
Qualitative Methods	20
Quantitative Methods	21
Statistical Analysis	22
RESULTS	23
I Histochemical Studies	23
Formaldehyde induced Fluorescence	23
Staining Reactions	28
Autofluorescence	34
Discussion	36
Silver Reactions	36

Other Staining Reactions	37
Fluorescence Reaction	37
II Consecutive Demonstration of Formaldehyde induced Fluorescence and Staining, Reactions in Enterochromaffin Cells	39
Results	39
Discussion	39
III Enterochromaffin Cells and 5 Hydroxytryptamine in the Duodenum	43
Correlation between the Number of Enterochromaffin Cells and the 5 Hydroxytryptamine Concentration	43
Material	43
Results	43
Mean 5 Hydroxytryptamine Content of the Enterochromaffin Cells	45
Distribution of the Enterochromaffin Cells and 5 Hydroxytryptamine in the Different Duodenal Layers	46
Material	46
Results	46
Enterochromaffin Cells and 5 Hydroxytryptamine in the Developing Duodenum	46
Material	46
Results	47
Discussion	48
Enterochromaffin Cells	48
5 Hydroxytryptamine	50
Correlation between the Enterochromaffin Cells and 5 Hydroxytryptamine	50
IV Experimental Studies	53
Effect of Reserpine on the Enterochromaffin Cells and 5 Hydroxytryptamine in the Duodenum	53
Reserpine Experiments	53
Guinea Pig	55
Qualitative Studies	55
Quantitative Studies	59
Rat	61
Qualitative Studies	61
Quantitative Studies	61
Effect of Cortisone on the Enterochromaffin Cells and 5 Hydroxytryptamine in the Duodenum	63
Material	63
Results	63
Discussion	63
Reserpine Studies	63
Cortisone Studies	65
SUMMARY	66
1 Histochemical Reactions of Enterochromaffin Cells	66
2 Enterochromaffin Cells and 5-Hydroxytryptamine in the Mammalian Duodenum	66
3 Experimental Studies	67
4 Conclusions	67
REFERENCES	67

## INTRODUCTION

Small cells which stained specifically brown after fixation with solutions containing dichromate were first described in the stomach of the dog and rabbit by Heidenhain in 1870. This kind of cells later called enterochromaffin cells were found by Toldt (1880) also in other species. Nicolas (1891) was the first to find enterochromaffin cells in the intestine. He was convinced that the enterochromaffin cells in the lizard were cells *sui generis*. Later it was generally stated by many other investigators that the enterochromaffin cells in the alimentary canal were a special cell type, although Ellenberger (1911) denied that they were autonomic cells and Eklof (1914) considered the enterochromaffin granules artifacts caused by an unsuccessful fixation.

In the literature enterochromaffin cells have many different names. Earlier they were often called after their investigators the Kultschitzky, Schmidt, Ciaccio or Masson cells. Because of the granular cytoplasm Kaufmann-Wolf (1911) introduced the term basal granular cells. Mostly however the cells were called after their staining properties. Schmidt (1905) introduced the term yellow cells. Kull (1925) chromaffin cells and Cordier (1926) chromo-argentaffin cells. The terms argentaffin, first used by Masson (1914), argyrophil (Hamperl 1932) and argentophil cells (Erspamer 1937) indicate the importance of silver reactions for demonstrating these cells. Ciaccio (1906, 1907) found that the staining properties of the chromaffin cells of the gut were similar to those of the medullary cells in the adrenal gland already commonly called chromaffin cells and introduced the term enterochromaffin cells. This term has been generally accepted and it is universally used today.

The histochemical properties of the enterochromaffin cells are quite dissimilar to those of the adjacent epithelial cells in the alimentary canal. This dissimilarity has given reason for many theories about the chemical structure of the enterochromaffin substance (Ciaccio 1907, Hamperl 1925, Cordier and Lison 1930, Gomori 1948). It was suggested by Erspamer and his associates (Erspamer 1940a, Vialli and Erspamer 1940, Asero *et al.* 1952, Erspamer and Asero 1952a, b) that 5-hydroxytryptamine is the specific substance in the enterochromaffin cells. This theory has been supported by many comparative and experimental investigations.

However, there are several unsolved problems concerning the enterochromaffin cells and the intestinal 5-HT, as will become apparent from the following literature review. Therefore the present study was undertaken to elucidate the enterochromaffin cell system in different mammals and its correlation to 5-hydroxytryptamine.

Other Staining Reactions	37
Fluorescence Reaction	37
II Consecutive Demonstration of Formaldehyde induced Fluorescence and Staining Reactions in Enterochromaffin Cells	39
Results	39
Discussion	39
III Enterochromaffin Cells and 5 Hydroxytryptamine in the Duodenum	43
Correlation between the Number of Enterochromaffin Cells and the 5-Hydroxytryptamine Concentration	43
Material	43
Results	43
Mean 5 Hydroxytryptamine Content of the Enterochromaffin Cells	45
Distribution of the Enterochromaffin Cells and 5-Hydroxytryptamine in the Different Duodenal Layers	46
Material	46
Results	46
Enterochromaffin Cells and 5-Hydroxytryptamine in the Developing Duodenum	46
Material	46
Results	47
Discussion	48
Enterochromaffin Cells	48
5-Hydroxytryptamine	50
Correlation between the Enterochromaffin Cells and 5 Hydroxytryptamine	50
IV Experimental Studies	53
Effect of Reserpine on the Enterochromaffin Cells and 5 Hydroxytryptamine in the Duodenum	53
Reserpine Experiments	53
Guinea Pig	55
Qualitative Studies	55
Quantitative Studies	59
Rat	61
Qualitative Studies	61
Quantitative Studies	61
Effect of Cortisone on the Enterochromaffin Cells and 5 Hydroxytryptamine in the Duodenum	63
Material	63
Results	63
Discussion	63
Reserpine Studies	65
Cortisone Studies	66
SUMMARY	66
1. Histochemical Reactions of Enterochromaffin Cells	66
2. Enterochromaffin Cells and 5-Hydroxytryptamine in the Mammalian Duodenum	66
3. Experimental Studies	67
4. Conclusions	69
REFERENCES	69

## INTRODUCTION

Small cells which stained specifically brown after fixation with solutions containing dichromate were first described in the stomach of the dog and rabbit by Heidenhain in 1870. This kind of cells later called enterochromaffin cells were found by Toldt (1880) also in other species. Nicols (1911) was the first to find enterochromaffin cells in the intestine. He was convinced that the enterochromaffin cells in the lizard were cells *sui generis*. Later it was generally accepted by many other investigators that the enterochromaffin cell in the enteric canal were a special cell type although Ellenberger (1911) denied this. E. G. autonomic cells and Eklof (1914) considered the enterochromaffin cells as facts caused by an unsuccessful fixation.

In the literature enterochromaffin cells have many different names but they were often called after their investigators the Kultschitzky Schmidt, Ciaccio or Masson cells. One of the first to use the term "basal ganglia" Kaufmann Wolf (1911) introduced the term "basal ganglia" cells. Mostly however the cells were called after their stain reaction. Ciaccio (1905) introduced the term "yellow cells", Kull (1923) "chromaffin cells" and Lordier (1926) "chromo argentaffin cells". The term "argyrophil" first used by Masson (1914) "argyrophil" (Hampert 1922) and "argentophil" cells (Erspamer 1937) indicate the specific reactions for demonstrating these cells. Ciaccio (1906) 1907 and 1908 staining properties of the chromaffin cells of the gut were compared with the medullary cells in the adrenal gland, already commonly called "chromaffin" and introduced the term "enterochromaffin cell". This term is generally accepted and it is universally used today.

The histochemical properties of the enterochromaffin cells are similar to those of the adjacent epithelial cells in the alimentary canal. Ciaccio has given reason for many theories about the chemical nature of the chromaffin substance (Ciaccio 1907, Hampert 1923, Lordier 1926, Gomori 1948). It was suggested by Erspamer and his colleagues (Erspamer 1940a, Vialli and Erspamer 1940, Asero *et al.* 1952, Erspamer 1952a, b) that 5 hydroxytryptamine is the specific substance in the chromaffin cells. This theory has been supported by many comparative studies.

However there are several unsolved problems concerning enterochromaffin cells and the intestinal 5 HT as will become apparent in the literature review. Therefore the present study was undertaken to follow the enterochromaffin cells, term in different mammals and the 5 hydroxytryptamine.

## REVIEW OF LITERATURE

### THE ENTEROCHROMAFFIN SYSTEM

In a strict sense the enterochromaffin system comprises the enterochromaffin cells (henceforth to be referred to as EC) of the gastrointestinal tract and of all glandular elements of intestinal origin (Erspamer 1958). Earlier it was generally accepted that the EC were morphologically and chemically identical in all the intestinal parts of different animal species. Therefore it was assumed that also the staining properties of the EC were similar. Clara (1933a, b) however showed remarkable differences in various staining reactions between EC in different vertebrate classes. With the aid of two silver reactions Erspamer (1937, 1938) and Viall and Erspamer (1939) divided the cells of the enterochromaffin system into four types: 1) pre-enterochromaffin non-argyrophil cell, 2) pre-enterochromaffin argyrophil cell, 3) enterochromaffin cell proper, 4) empty enterochromaffin cell. This classification represented various successive stages in the maturation of the EC. True EC could be stained with all the specific staining reactions such as argyrophil, argentaffin, ferric ferri-ferrocyanide, diazo coupling and indophenol reactions. On the other hand the argyrophil cells stained specifically only by the silver impregnation method in which reaction the extraneous reducing substance caused the blackening of the EC. In the argentaffin reaction the EC themselves were able to reduce the silver solutions. The first and last stages of the cycle could not be defined histochemically.

The true EC were stated to contain a specific substance 5-hydroxytryptamine whereas only its precursors were found in the argyrophil stage (Erspamer 1958). This theory was supported by Monesi (1960a) who demonstrated that the argyrophil cells preceded EC in the intestine of the chick embryo. It was noted by Hellveg (1952) and Hammerl (1952) that among the human intestinal EC there were 50% argyrophil and 5% argentaffin cells and 45% EC showing both staining properties. With adequate methods Singh (1963, 1964a) could find in the human intestine no EC with argentaffin properties only.

Thus confirmed Erspamer's theory that there were only two histochemically demonstrable types of EC. The difference between argyrophil and argentaffin cells was demonstrated in the electron microscopy by Toner (1964). The granules of the argentaffin cells varied more in shape and size and they were larger and coarser than the granules in the argyrophil cells. Ratzenhofer and Leb (1965) further showed that the argentaffin granules were osmophilic but the argyrophil granules osmophobic.

The concept of the enterochromaffin system was expanded by Erspamer (1958, 1961). He called EC all such cells also the cytoplasmic granules of which could be stained after the formalin fixation with argentaffin, chromaffin, diazo coupling and alkaline thionin reactions and that showed a formalin induced fluorescence. Cells of this kind were found in the mammalian urethra

birds (Quaroni 1956) and reptiles (Valli and Casati 1958) in the cutaneous glands of amphibians (Valli 1955) in the lizard oviduct and the frog urinary bladder (Erspamer 1958) in the venous glands of scorpions (Adam and Weiss 1959) in the posterior salivary glands of octopods and in the hypobranchial body of some molluscs (Erspamer 1958) and in the nematocysts of some coelenterates (Hamon 1955). All the cells of the enterochromaffin system contained 5-hydroxy tryptamine or related substances and therefore Erspamer included also the mast cells of the rat and mouse into this system.

On the other hand Feyrter (1953) included the EC of the intestinal tract in another system the so called »helle Zellen« organs. Also this classification was introduced on the ground of the staining properties of EC granules. The scattered clear cells were derived from the inner and outer epithelium of the body and apparently had an endocrine function.

In the present study the EC in the intestinal tract will be mainly discussed.

## ENTEROCHROMAFFIN CELLS IN DIFFERENT SPECIES

EC are to be found in the alimentary canal of all the classes of vertebrates investigated and also in some groups of invertebrates (Erspamer 1958).

Most studies have been made with mammals. In adult man the EC were first described by Zimmermann (1898) and in the nursing by Bloch (1903). Kulcschitzky (1897) and Moller (1899) discovered EC in both the small and the large intestine of some domestic animals. In all the adult mammals investigated the EC were found in every part of the intestinal tract from the stomach to the anus (Muthmann 1913, Tang 1922, Kull 1925, Hamperl 1925, Cordier 1926, Tehver 1930, Clara 1933a, Jacobson 1939, Dawson 1945). Studies on embryos are not so numerous but they all show that EC are to be found already during embryonic life. In the human intestinal tract the EC appear between the fifth and sixth month of gestation according to Kull (1925) and between the sixth and twelfth week according to Cordier (1926) and Cole and McEalen (1962). In the intestine of domestic animals the EC were found during the first half of gestation (Tehver 1930). Faustini (1955) demonstrated EC in the fifth week of intrauterine life in the calf embryo.

The existence of EC in the intestinal tract of most species of birds has been stated by Muthmann (1913), Kull (1925) and Clara (1926a, b). In chick embryos of 14–16 days Monesi (1960a, b) found intestinal EC both in the normal and tissue cultured intestine. In the reptilia and amphibia the EC were found almost in every species investigated (Kull 1925, Cordier 1926, Valli 1929, Toro 1930, Clara 1932, 1933a). Only some species of fish showed EC (Kull 1925, Cordier 1926, Rogosina 1928, 1930). De Filippi (1930) found no EC in the teleosts but recently the existence of the argyrophil cells in this fish group was demonstrated by Erspamer (1958).

## DISTRIBUTION OF THE ENTEROCHROMAFFIN CELLS

According to Schmidt (1905) and Cordier (1926) the distribution of the EC was quite even in different parts of the intestine. However, Hamperl (1925) reported that EC were most numerous in the duodenum and that there was a steady decrease in their number in the caudal direction of the intestine. This observation was later confirmed by precise quantitative estimations made by Tehver (1930) in the pig, horse, dog and cat, by Hoeschen (1937) in the guinea pig and by Munch (1939) in the rat. It was noted by Hoeschen (1937), Vetter (1937, 1938) and Munch (1939) that in the guinea pig and rat EC were most numerous in the duodenal part near the pylorus and that their count strikingly decreased already in this part of the small intestine in the anal direction. Only in the cow were large numbers of EC found in the rectum (Tehver 1930). In the



coecum of some mammals EC were relatively numerous (Kull 1925 Cordier 1926 Tehver 1930) Also in the human appendix the number of EC was considerably high (Sprafke 1927 Masson 1928 Schack 1932)

In other vertebrates Kull (1925) and Clara (1926a) observed a similar duodenal predominance in the distribution of the EC except for some bird and fish species which showed an increasing number of EC in the caudal direction

In the stomach the demonstration of the EC in several species was difficult before the argyrophil reaction was used Dawson (1944 1945) and Sharples (1945a b) showed that EC were rather numerous also in the stomach of mammals especially in the fundic region In the oesophagus Kull (1925) and Feyrter (1934) found very few EC situated always near the cardia but according to Tehver (1930) Dawson (1945) and Sharples (1945a) the EC were absent in the oesophagus as also in the squamous stratified epithelium of the stomach

It has been generally observed that even in the same species the individual variations in the EC count in the intestinal tract are great Hallier (1937) Klemm (1937) Vetter (1938) and Schumann (1939) showed that the number of EC was distinctly greater in adult female guinea pigs rats and mice than in male animals Schumann further observed a steady increase of the EC count in the guinea pig during gestation Since the time of Heidenhain (1870) it has often been reported that the amount and kind of food have a great influence on the number of stained EC

In the alimentary canal of all vertebrates the EC were diffusely distributed among the epithelial cells (Clara 1933a) Sometimes great EC accumulations were observed The most numerous EC were found in the mucosa particularly in the basal parts Also in Brunner's glands some EC were often seen

## MORPHOLOGY OF THE ENTEROCHROMAFFIN CELLS

The coarse cytoplasmic granularity of the EC was observed already by Heidenhain (1870) This property made EC easily distinguishable from the other gastrointestinal cells According to Hamperl (1925) the average size of the EC was 21—24 micra in length and the cell basis was 8—12 micra in breadth even though there were variations in different animals Also the shape of the EC varied considerably in the different parts of the mucosa

Electron microscopy studies on mammalian EC have been recently made by Christie (1955) Helander (1961) and Wetzstein *et al* (1962) They all observed that the cytoplasm of the EC contained numerous more or less evenly dispersed oval or circular bodies The specific granules surrounded by a membrane had an irregular internal structure Their average size was 0.2—0.3 micra The granules were mostly situated in the basal part of the EC and were often absent in the apical part The endoplasmic reticulum was well developed particularly above the nucleus The EC were in direct contact with the basal membrane and in some cells the ciliated free cytoplasm extended into the intestinal lumen

## HISTOCHEMICAL REACTIONS OF ENTEROCHROMAFFIN CELLS

### *Fixation of Enterochromaffin Cells*

Already in the early studies formalin was generally used for the fixation of EC Hamperl (1925 1927) showed that formalin was necessary for the preservation of the staining properties of EC Good results were obtained with pure aqueous solutions of formalin or with Bouin's or Orth's fluids In different species the optimum composition of the fixative has been found

to vary considerably Clara (1933b) demonstrated that Bouin's fluid was especially effective in birds but not so good in amphibians. After 5-hydroxytryptamine (henceforth to be referred to as 5-HT) was found in the EC Barter and Pearse (1953) and later Holcenberg and Benditt (1961) showed that synthetic 5-HT and formaldehyde condensed to an unconjugated tetrahydro-4-carboline derivative. This compound showed an intense yellow fluorescence in ultraviolet light. The dry formaldehyde vapour also condensed with 5-HT to this unsaturated derivative (Corrodi and Hillarp 1963). Holcenberg and Benditt (1961) demonstrated that the carboline derivative was highly insoluble in water and in various organic solvents but dissolved readily in 0.1 N HCl. This derivative is the reason for the specific staining properties of the fixed EC.

It was found quite early that fixatives containing alcohol, acids or sublimate fixed EC less effectively (Hamperl 1925). Using paper chromatography Eder *et al.* (1962) demonstrated that 5-HT and formaldehyde produced no condensation derivative in acids and no water insoluble derivatives in alcoholic solutions. In neutral water solutions four condensation products were formed with different chromatographic properties. In striking contrast to all the other specific staining reactions in the EC the argyrophil reaction was positive also in tissues fixed in fixatives containing alcohol (Dawson 1944; Hellweg 1952; Hamperl 1952; Eder *et al.* 1962). Eder *et al.* further showed that also the argyrophil reaction was negative in EC fixed in pure alcohol.

Earlier it was generally assumed that the granules in the EC were not original but artifacts produced by formalin fixation. This hypothesis was denied by Christie (1955). He demonstrated that the dark EC granules fixed in an osmium tetroxide solution without any formalin treatment were beautifully seen in electron microscopy.

### Fluorescence Reaction

Eros (1932) was the first to find golden yellow fluorescent cells in the intestine and some endocrine glands fixed in a formalin solution. Only formalin made this fluorescence possible but the nature of the fluorogenic material remained obscure. Jacobson (1939) measured the fluorescence spectra in human carcinoid tumour cells. He also studied the influence of temperature, oxidation, alkaline solutions, enzymes and illumination on the properties of the fluorescence. In general this 5-HT fluorescence was very permanent. Eranko (1951, 1952) was the first to observe that some medullary cells in the adrenal gland showed a strong greenish fluorescence in formalin fixed sections which was later (Eranko 1955a) shown to be due to noradrenaline. Shepherd *et al.* (1953) studied several amines by chromatography after formalin treatment and found that the fluorescence characteristics of synthetic 5-HT, bufotenine and bufotenidine were quite similar. It was noticed by Barter and Pearse (1953, 1955) that the fluorescence of synthetic 5-HT and that of the EC in the guinea pig was of a similar yellow colour. They first treated the freeze-dried tissues in damp formaldehyde vapour but the method was not very successful because of the easy diffusion of 5-HT. Lagunoff *et al.* (1961) and Eranko (1961) noticed that the dry formaldehyde vapour converted several monoamines into strongly fluorescent compounds. Based on a similar observation Falck and Torp (1961) developed the method suitable for the distinct histochemical demonstration of fluorescent monoamines in freeze-dried tissues.

The mechanism of the formaldehyde induced fluorescence reaction has been recently studied thoroughly particularly by using model systems and the chemical as well as the histochemical background is now well understood (Falck *et al.* 1962; Corrodi and Hillarp 1963, 1964; Corrodi *et al.* 1964; Corrodi and Jonsson 1965). The catecholamines were converted into isoquinoline derivatives which had a green fluorescence colour, sometimes a yellowish tinge. 5-HT and 5-hydroxytryptophan reacted principally in the same way. Only those indolic compounds with a tryptamine side chain and with an unsubstituted 2 position are condensed with formaldehyde

(Falck and Owman 1965) Only the indoles with 5-hydroxy and 5-methoxy groups formed condensation products which showed an intensive yellow fluorescence. In the activation spectra the main peaks for catecholamines and indole derivatives were in the same region whereas the main emission peaks were at 480 nm for catecholamines and at 510 nm for 5-HT. This difference between the emission peaks made it possible to distinguish the fluorescence of the catecholamines from that of the indolic derivatives. With this method Bertler *et al* (1964) obtained a very strong fluorescence of 5-HT in the pineal gland of the rat. Also very small amounts of 5-HT in the brain stem were demonstrated with this method (Dahlstrom and Fuxe 1964). Lagunoff *et al* (1961) found in mast cells of the rat tongue a strong yellow fluorescence after paraformaldehyde treatment. The uptake of several monoamines by mesenteric mast cells of the mouse was beautifully demonstrated by fluorescence microscopy (Eranko and Kauko 1965). Only Norberg (1964) and Hammarstrom *et al* (1966) have shown that the EC exhibit a strong yellow fluorescence in the small intestine of the cat, mouse and rat.

### *Silver Reactions*

**Argentaffin Reaction** — Masson (1914) was the first to observe that Fontana's ammoniacal silver solution stained the EC blackish in the human intestine after formalin fixation. The method was simplified by Hamperl (1925). This 'Masson-Hamperl's method' has been the most commonly employed one for the demonstration of the EC. Modifications of the argentaffin reaction were introduced by Gomori (1948) and Burtner and Lillie (1949) but the results obtained were essentially similar to those obtained with the original method.

In the argentaffin reaction the formalin fixed EC themselves were capable of reducing ammoniacal silver solutions into metallic silver (Hamperl 1925). Lison (1931) observed the low chemical specificity of this reaction. He pointed out that it was given positive by several biological substances among which phenols were the most important. According to Glick (1949) also many other reducing substances made the reaction strongly positive. Pearse (1953) considered the argentaffin reaction specific for aldehydes which opinion however was contradicted by Lillie (1954b).

**Argyrophil Reaction** — In silver impregnation methods the precipitate is produced by an extraneous reducing agent. The precipitate is first formed in 'argyrophil' sites of a tissue. Such silver impregnation methods have mainly been used for the staining of nervous tissue but Hasegawa (1923) developed a modification of his own and showed that the EC in the human small intestine and appendix had argyrophil properties. Thereafter many other modifications designed for nervous tissues have been used for the demonstration of the EC, such as Agduhr's method (Danisch 1924), Brieschowsky's method (Hamperl 1925), Masson's method (Masson 1928), Gros-Schulze's method (Hamperl 1932) or Singh's (1964b) method. All these methods especially those in which large tissue pieces are impregnated were often reported to be capricious. Bodian (1936, 1937) developed a more reproducible method. Instead of the silver nitrate solution the organically bound silver compound silver tartrate (S<sub>2</sub>) was used. Dawson and Barnett (1944) found this method particularly suitable for the demonstration of the EC. Nowadays this method is most commonly used for the staining of the 'argyrophil' cells.

Hamperl (1925) already pointed out the principal difference between the argentaffin and argyrophil reactions. The reducing ability of the enterochromaffin substance makes the EC directly positive in the argentaffin reaction but why the argyrophil reaction becomes positive only after the addition of an extra reducer is obscure. Although the silver impregnation method was chemically highly unspecific, it was selective for many morphological structures (Gomori 1948).

## Diazo Coupling Reaction

Cordier and Lison (1930) were the first to use fresh diazo granules for the reaction. Clara (1932-1934) obtained similar results with both fresh and stable diazo granules. Lison (1931) and Clara (1932) further observed that the EC reacted with *p*-nitroaniline. It was noted by Pearse (1960) and Lillie *et al* (1961) that some EC were stained brown, 40-52. The pH optimum was, however, clearly on the alkaline side. The detection of specifically stained organelles from the background depended on the efficiency of the stain. Lillie *et al* (1961) demonstrated that the best results were obtained with fresh diazo granules. Also many stable nitroanilines, nitroanilines, and other aromatic amines were used.

The chemical basis of the diazo coupling reaction was studied by Cordier and Lison (1930). The reaction was given by aromatic amines and pH and by the presence of a hydroxyl group must be unsubstituted. At least one of the para positions must be free. The hydroxyl group must also be free. The reaction was on the EC as well as on fresh diazo granules and depended on the structure of the stain and the reaction.

## Indophenol Reaction

Gibbs (1927) found that phenols reacted with 2,6-dichloroquinone in a buffer solution. The reaction product was an indophenol derivative the color of which depended on the reacting phenol. Gomori (1948) was the first to use this reaction with histology. According to him the reaction was specific for catechol compounds and *para* substituted phenols. The Griba reaction has been used for demonstrating EC. It was shown by Lillie and Gerner (1957) that the reaction was less sensitive in the EC and carcinoid tumours. Moderately positive results were obtained by Lillie *et al* (1957) in the duodenum and stomach of the rabbit and by Gerzels (1961) in the intestine of an insectivorous animal.

Lison (1931) used a dimethyl *p*-phenylenediamine salt as a stain in the presence of an oxidizing agent. In the vertebrate EC this reaction produces a brown stain which is not commonly used.

## Ferric Ferricyanide Reaction

The ferric ferricyanide reaction was introduced by Cordier and Lison (1930) into histology. Ferricyanide was reduced to ferrocyanide by tissue components and there after it was converted in the presence of ferric ions to insoluble Prussian blue. Gomori (1948) first noticed that this reaction stained also the EC granules. Lillie (1954) found that the 15:1 in contrast to the earlier 6:1 stained the EC more efficiently.

The ferric ferricyanide reaction is chemically quite unspecific and all reducing substances give a positive reaction (Lillie and Burton 1953; Lillie 1954).

## Ferric Chloride Reaction

It was demonstrated by Cordier and Lison (1930) that all the proteins reacted in a dilute ferric chloride solution. The method is seldom used for the detection of the EC granules because of its weak staining ability. Therefore Cordier and Lison did not dare to speak of a positive reaction in the EC. However, Clara (1933a, b) observed that the EC in mammals and birds turned greenish whereas in reptilia and amphibia they were brown.

## Ninhydrin Reaction

Jepson and Stevens (1953) discovered a specific test for the tryptamines which did not contain substituents either at position 2 or at the nitrogen atom. In ultraviolet light synthetic compounds of this kind gave on dried paper a strongly blue green fluorescence after treatment with ninhydrin in acetone containing glacial acetic acid. Holcenberg and Benditt (1959-1961) introduced this principle into histochemistry. They found that after formalin fixed sections of the guinea pig duodenum had been treated in the ninhydrin vapour in vacuum the EC granules were stained orange red. This colour was given only by indolethylamine compounds. On the indole ring there ought to be a free 2 position, a free  $\alpha$ -carbon atom as well as an amine group on the ethylamine side-chain. The compounds of the tetrahydro-4-carboline derivatives with unsubstituted 3-, 4- and 5 positions and substituted pyridic ring gave a positive reaction. The sensitivity of the reaction was similar to that of the argentaffin and diazo coupling reactions. According to Barka and Anderson (1963) the ninhydrin reaction is one of the most specific for the enterochromaffin substance.

## Oxidative Agents

Heidenhain (1870) observed that the unfixed EC granules turned yellow in a potassium dichromate solution. Later it was demonstrated that many other oxidizing agents such as nitric acid, bromine water, potassium permanganate and iodates produced a yellow colour in the EC (Patzelt 1936, Gomori 1948). Recently it was noted by Friksen *et al* (1960) and by Smuckler *et al* (1960) that synthetic 5-HT was converted with the aid of oxidants to polymers and brown pigments. The polymers were formed by dehydrogenative coupling through the aromatic rings of the molecule. They further suggested that obviously also the enterochromaffin substance was converted by oxidizing agents to polymers rather than to the quinhydrone type of substances as postulated by Gérard *et al* (1930).

## Other Reactions

Clara (1935) studied the chemical nature of several usual dyes which stained the EC granules. These dyes contained in their molecule hydroxyl groups arranged in a catechol manner. In dilute solutions of haematoxylin, gallocyarin, gallamine blue, celestin blue, alizarin, cyanin R or naphthazarin the EC granules were stained quite specifically. According to Clara, the dyes were absorbed by the EC granules. In contrast to this, Lillie (1961) suggested that the reaction was given positive by an open phenolic group in the enterochromaffin substance. He found no reaction in tryptamine models.

Quatrel's reagent (containing ammonium molybdate and glacial acetic acid) for demonstrating *o*-diarylbenzene derivatives was shown by Clara (1933a, b) to be positive in the EC of mammals and birds and negative in those of reptilia and amphibia.

The chemistry of Mallory-Heidenhain's azocarmine staining reaction is still unknown. Gomori (1946, 1948) demonstrated that also this reaction specifically stained the EC granules red or purplish, especially in human carcinoid tumours.

It was demonstrated by Christie (1954) that the EC granules gave a positive acid haematein reaction. Pearse (1960) however showed that the reaction was weak in the EC of man and guinea pig. Besides all the other reactions for phospholipids were negative in the EC.

Pearse (1956) found that the formalin fixed EC were stained reddish brown in the alkaline thioindoxyl reaction. The  $\beta$ -carboline derivative of 5-HT and formaldehyde was oxidized with iodine to the quinonimine. This derivative reacted in an alkaline solution with unsubstituted or substituted thioindoxyls forming thioindigoid dyes.

## CHEMICAL CHARACTER OF THE ENTEROCHROMAFFIN GRANULES

The staining reactions described have caused many theories concerning the chemical nature of the enterochromaffin granules

### *Catechol Theory*

Ciaccio (1907) demonstrating that the EC and adrenal medullary cells were stained quite similarly yellow in dichromate solutions suggested that adrenaline was the principal substance of the EC. This hypothesis was supported by Masson's (1914) experiments with the argentaffin reaction. Hamperl (1925) showed that the silver and chromaffin reactions were caused by a *brenzcathecin* derivative. This theory was supported by several other investigations (Cordier and Lison 1930, Lison 1931, Clara 1932, 1933a, 1934, Clara and Canal 1932, Vialli and Erspamer 1933). It was demonstrated that the diazo coupling and indophenol reactions were given by phenols in alkaline solutions. The monophenols and polyphenols were excluded by chemical experiments. Earlier Verne (1923) had shown that only *para*- and *ortho*-diphenols were capable of giving a positive chromaffin reaction. Positive ferric chloride and Quastel reactions suggested that the enterochromaffin substance ought to be an *ortho*-diphenol. This suggested catechol substance an *o*-dihydroxybenzene derivative had a simple unknown side-chain in the *para* position to a hydroxyl group.

### *Pteridine Theory*

From the argentaffin and diazo coupling but especially from the positive murexide reaction in the EC Jacobson (1939) concluded that the enterochromaffin granules contained a purine compound. Jacobson's positive Hilum and Schiff reactions in the EC which observations it was not possible to confirm later (Lillie 1961) suggested that the enterochromaffin substance was a carbohydrate compound. Jacobson made also spectrographic studies and observed the fluorescence properties to be quite similar in xanthopterin and in carcinoid enterochromaffin substance. Based on these and other chemical comparisons Jacobson and Simpson (1946) and Jacobson (1954) claimed that the enterochromaffin substance was a pteridine compound. This hypothesis was shown to be incorrect by Gomori (1948) and Pearse (1960).

### *Resorcinol Theory*

Gomori (1948) showed that the catechol theory had three fallacies. Catechols and their derivatives were more strongly reducing agents than the enterochromaffin substance. Secondly, the positive indophenol reaction was against a *para*-substituted phenol. Thirdly, in the diazo coupling reaction there were many exceptions in the staining ability of *ortho*- and *para*-diphenols. Gomori compared the staining properties of various substances with those of the EC granules in model systems. From the results obtained with argentaffin, diazo coupling and Gibb reagents he concluded that the enterochromaffin substance was a derivative of *metadiphenol* (resorcinol) rather than an *ortho*-diphenol as previously suggested. Gomori's theory was supported by Lillie *et al.* (1953). Recently the 5 HT theory has abolished the resorcinol theory although Gomori (1954) claimed that it was impossible to show if 5-hydroxyindole, resorcinol or some unknown chemical compound was the main enterochromaffin substance in the diazo coupling reaction.

### *5-Hydroxytryptamine Theory*

The greatest advances in studies on the enterochromaffin system were made by Erspamer and his associates during the years 1940–1952. From the stomach mucosa of the rabbit Erspamer

## Ninhydrin Reaction

Jepson and Stevens (1953) discovered a specific test for the tryptamines which did not contain substituents either at position 2 or at the nitrogen atom. In ultraviolet light synthetic compounds of this kind gave on dried paper a strongly blue green fluorescence after treatment with ninhydrin in acetone containing glacial acetic acid. Holcenberg and Benditt (1959, 1961) introduced this principle into histochemistry. They found that after formalin fixed sections of the guinea pig duodenum had been treated in the ninhydrin vapour in vacuum the EC granules were stained orange red. This colour was given only by indolethylamine compounds. On the indole ring there ought to be a free 2 position, a free  $\alpha$ -carbon atom as well as an amine group on the ethylamine side-chain. The compounds of the tetrahydro-4-carboline derivatives with unsubstituted 3-, 4- and 5-positions and substituted pyridic ring gave a positive reaction. The sensitivity of the reaction was similar to that of the argentaffin and diavo coupling reactions. According to Barka and Anderson (1963) the ninhydrin reaction is one of the most specific for the enterochromaffin substance.

## Oxidative Agents

Heidenhain (1870) observed that the unfixed EC granules turned yellow in a potassium dichromate solution. Later it was demonstrated that many other oxidizing agents such as nitric acid, bromine water, potassium permanganate and iodites produced a yellow colour in the EC (Patzelt 1936, Gomori 1948). Recently it was noted by Eriksen *et al* (1960) and by Smuckler *et al* (1960) that synthetic 5-HT was converted with the aid of oxidants to polymers and brownish pigments. The polymers were formed by dehydrogenative coupling through the aromatic rings of the molecule. They further suggested that obviously also the enterochromaffin substance was converted by oxidizing agents to polymers rather than to the quinhydrone type of substances as postulated by Cérard *et al* (1930).

## Other Reactions

Clara (1935) studied the chemical nature of several usual dyes which stained the EC granules. These dyes contained in their molecule hydroxyl groups arranged in a catechol manner. In dilute solutions of haematoxylin, gallocyain, gallamine blue, celestin blue, alizarin, cyanin R or naphthazarin the EC granules were stained quite specifically. According to Clara the dyes were absorbed by the EC granules. In contrast to this Lillie (1961) suggested that the reaction was given positive by an open phenolic group in the enterochromaffin substance. He found no reaction in tryptamine models.

Quastel's reagent (containing ammonium molybdate and glacial acetic acid) for demonstrating *o*-di-*ortho*-benzene derivatives was shown by Clara (1933a, b) to be positive in the EC of mammals and birds and negative in those of reptiles and amphibians.

The chemistry of Mallory-Heidenhain's azocarmine staining reaction is still unknown. Gomori (1946, 1948) demonstrated that also this reaction specifically stained the EC granules red or purplish, especially in human carcinoid tumours.

It was demonstrated by Christie (1954) that the EC granules gave a positive acid haematein reaction. Pearse (1960) however showed that the reaction was weak in the EC of man and guinea pig. Besides all the other reactions for phospholipids were negative in the EC.

Pearse (1956) found that the formalin fixed EC were stained reddish brown in the alkaline thionin xyl reaction. The 3-carboline derivative of 5-HT and formaldehyde was oxidized with iodine to the quinazolinone. This derivative reacted in an alkaline solution with unsubstituted or substituted thionin xyls forming thionindigo dyes.





## PROBLEMS OF THE PRESENT STUDY

In spite of the numerous separate investigations concerning the histochemical properties of the EC, no systematic comparison has been made between various mammalian species. Moreover the ferric ferricyanide diazo coupling and indophenol reactions have seldom been used for the demonstration of EC. The formaldehyde induced fluorescence in the EC has been reported only as an observation. The hypothesis that the EC count and the 5 HT concentration are in a direct correlation to each other in the intestinal tract is based on the separate investigations made by anatomists and pharmacologists. Both variables greatly depend on the environmental and racial conditions of the animals as well as on the techniques used for the quantitative determinations. In order to avoid uncertain estimations of the correlation between the EC and 5 HT it is necessary to determine both quantities in adjacent tissue pieces. This kind of determinations have hardly been made earlier.

The main problems of the present study were formulated as follows:

- Are the histochemical properties of the EC similar in the different mammals?
- How effective are the different reactions in demonstrating the mammalian EC?
- Are there one or several kinds of histochemically demonstrable EC?
- Is there a correlation between the number of EC and the 5 HT content in the normal mammalian duodenum?
- What is the effect of reserpine and cortisone on the EC and on the 5 HT content?

## MATERIAL

The material consisted of the small intestines of 32 sheep, 26 cows, 26 pigs, 18 horses, 82 guinea pigs, 19 mice, 176 rats and 9 rabbits. Only females were used.

The sheep, pigs and horses of Finnish domestic breed and the cows of Ayrshire breed were obtained from the municipal slaughterhouse of Helsinki. The guinea pigs and white rabbits of an unknown strain were bred at Munkkila farm (Orion Oy). The white mice of an unknown strain and the white rats, descendants of the Sprague Dawley strain, were bred in the colony of the Department of Anatomy.

The rats varied in age from 1–2 hours after birth to adult. Nine of the guinea pigs were newborn, all the other animals were adult. The sheep were 2–4, the cows 3–10, the horses 5–15 and the rabbits 1–2 years old, the pigs 6–8 and the mice 4–6 months, the adult guinea pigs and rats 3–6 months of age.

Before slaughtering the animals were kept in stables for 1–4 days at room temperature. The sheep received 1 kg, the cows 3 kg and the horses 5 kg of hay and the pigs 1 kg of oats per day. The guinea pigs were allowed to eat hay, swedes and «guinea pig pellets» (3.3% raw fat, 22.1% raw protein, 7.0% raw fibre, 44.8% nitrogen-free extractable substances, 9.1% ash and 13.7% water made by Orion Oy). The mice and rats were fed on a standard cube diet (3.5% raw fat, 24.0% raw protein, 5.5% raw fibre, 43.5% nitrogen-free extractable substances, 13.0% ash and 10.5% water made by Keskosuusliike Hankkija). The rabbits were fed on hay, oats and swedes. All the animals were allowed to drink water *ad libitum*; the laboratory animals had also free access to food.

The sheep were killed by a blow on the head, the cows and horses by shooting in the head and the pigs by electrocution. The laboratory animals were killed by a blow on the neck without anaesthesia. The duodenal parts were removed in the slaughterhouse from the sheep within 2–5, from the cows and pigs within 10–15 and from the horses within 20 minutes after killing. From the laboratory animals the tissue pieces were taken immediately after death.

The intestinal pieces from the sheep, cows, pigs, horses and rabbits were always taken near the pylorus. The pieces were cut into two parts: one for the freeze-drying procedure and the usual staining reactions and one for the 5-HT determination. From the guinea pigs, mice and rats the proximal third of the duodenum was removed. The middle portion of this duodenal third was used for the freeze-drying procedure or the histochemical staining reactions; the remaining parts for the 5-HT determination. The pieces used in the histochemical reactions were immediately put into fixatives. The pieces for the freeze-drying procedure were cooled in liquid nitrogen, kept in it for less than 2 hours and then transferred into a freeze-drying apparatus or stored with solid carbon dioxide at  $-78^{\circ}\text{C}$  for 1–7 days. The duodenal pieces for the 5-HT determination were kept for 10–20 minutes in a thermos box at  $\pm 0^{\circ}\text{C}$ .

## METHODS

### QUALITATIVE METHODS

A solution of 80% alcohol (90 ml) 99–100% glacial acetic acid (5ml) and 37–40% w/v neutral formaldehyde (5ml) was used as fixative for demonstrating the argyrophil reaction in the EC. In all the other staining reactions the tissue pieces were fixed in 5% aqueous formaldehyde solution. In both cases the fixing time varied from 2 to 5 days. The fixed tissues were dehydrated in alcohol series and embedded through butanol in paraffin wax. The sections were cut at 5 micra. The stained sections were dehydrated in alcohol, cleared in xylene and mounted in Canada balsam.

The following staining techniques were used

**Argyrophil Reaction** — Bodians (1936) silver impregnation method was used. The sections were immersed in 1% Protargol S (Sterling Drug Inc.) at 37°C for 12–24 hours. Copper nails were placed on the bottom of the staining dish. As a reducing agent served a solution of hydroquinone (1 g), sodium sulphite (5 g) and distilled water (100 ml). The reducing time varied from 5 to 15 minutes. The final staining was carried out in 1–2% oxalic acid solution. Overstaining was carefully avoided.

**Argentaffin Reaction** — The Fontana solution was used (see Romeis 1948). The final solution had to be distinctly turbid and without the odour of ammonia. The solution was used after 24 hours. The staining procedure was carried out by the method of Hamperl (1925) for 1–3 days. The stained sections were washed in distilled water and fixed in 5% sodium thiosulphate solution for 1–2 minutes.

**Ferric Ferriyanide Reaction** — The reduction techniques described by Lillie (1954a) and Pearse (1960) were used. The latter technique was mainly followed because no essential difference was noticed between these methods. The mixture was always prepared immediately before use and it was used only once. The staining time varied from 5 to 15 minutes.

**Diazo Coupling Reaction** — Only stable diazotates were used. The sections were stained in 0.1 M veronal acetate buffer at pH 9.2. The buffer contained either 0.1% 5-nitroanisidine (Fast Red Salt B I.C.I. Ltd.) or 0.1% p-nitroaniline (Fast Red Salt GG I.C.I. Ltd.). The dye solutions were prepared immediately before use according to Pearse (1960) and no essential difference was observed between the used salts in demonstrating FC. The staining procedure lasted 2–10 minutes.

**Indophenol Reaction** — In principle the technique given by Pearse (1960) was used. A 0.1% solution of Gibbs reagent 2,6-dichloro-quinonechloroimide (E. Merck AC) was prepared in 0.1 M veronal acetate buffer at pH 9.2. The mixture was warmed five times in 70°C and shaken vigorously. The staining was carried out at room temperature for 1 to 10 hours.

**Formaldehyde induced Fluorescence**—In principle the techniques given by Falck and Torp (1961) and Eranko (1964) were followed. Small duodenal pieces were frozen with the aid of metal disc forceps precooled in liquid nitrogen (Eranko 1954) or dropped directly into liquid nitrogen. They were dried in vacuo at  $-35^{\circ}\text{C}$  to  $-45^{\circ}\text{C}$  for 1–4 days. After drying the pieces were treated with formaldehyde vapour from paraformaldehyde at  $+80^{\circ}\text{C}$  for 1–2 hours in a closed glass vessel. The tissues were then embedded in paraffin wax directly under vacuum or after immersion in xylene. The sections were cut at 5  $\mu$  and were deparaffinized with xylene on slides for fluorescence studies. Some sections were attached on slides with a thin layer of fresh egg white and were then dried for the deparaffinization with xylene. The fluorescence was registered by microphotography. The various staining reactions were carried out and the sections were then rephotographed.

For fluorescence studies Wild's monocular microscope was used. The light source was a high pressure mercury lamp HBO 200 (Osram). The light was filtered through Schott BG12 and BG23 filters and two Schott K2 heat absorbing filters. The stop filters in the tube were Leitz Euphos and Schott OG1. Sometimes a dark field condenser was used. The photographs were taken on Perutz 27 film. Exposure times varied between 10 and 120 seconds. All optical components were of glass.

**Autofluorescence Reaction**—The specimens were removed, frozen and dried in the same way as in the fluorescence reaction but they were not exposed to paraformaldehyde.

## QUANTITATIVE METHODS

**Number of EC**—Freeze-dried specimens treated with paraformaldehyde vapour were used for the determination of the EC count. Small intestinal pieces 1–2 mm in diameter and 3–4 mm in length were taken from the duodenum of the sheep, cow, pig, horse and rabbit. These specimens were cut in the longitudinal direction of the intestine opposite to the mesenterium. The specimens taken from the guinea pig, mouse and rat were about 3 mm in length and included the whole transverse section surface of the duodenum. From each specimen 6–8 paraffin sections were cut at 5  $\mu$ . In order to avoid inaccuracies due to EC accumulations a distance of 100–200  $\mu$  was always left between the different sections. All counts were made by the present writer using a magnification of 200–600 $\times$ . The EC in 5–8 sections were counted and the mean number of the EC per section was defined for each specimen.

Other sections immediately adjacent to the counted sections were cut at 10  $\mu$  and stained with haematoxylin-eosin (see Romeis 1948). The magnified images (34 or 52 $\times$ ) were projected and drawn on paper. All drawings were measured with a planimeter (Eranko 1955b). The mean volume ( $\text{mm}^3$ ) of each specimen was defined. With the aid of these mean numbers and mean volumes the EC count per 1  $\text{mm}^3$  of duodenum was determined for each specimen. The values obtained were corrected according to the formula of Floderus (Eranko 1955b). The diameter of the fluorescent FC was determined from microphotographs (887 $\times$ ) for each species and each age group. The mean diameter defined from 60–104 EC in each case was the mean proportional of the product of the long axis of the EC and their largest breadth perpendicular to it. The mean vertical height of the smallest fluorescent structures that could be identified as EC was determined from 20 fluorescent bodies in microphotographs.

It was found that there were no EC in the muscularis interna and externa of the sheep, cow, pig and horse. Therefore tissue pieces containing only submucosa and mucosa were taken from these animals to facilitate successful freeze-drying. For each animal the relative proportion of the united muscular and the submucous mucous layers was determined in formalin fixed sections.

stained with haematoxylin eosin. This proportion was taken into consideration when determining the EC count in 1 mm<sup>3</sup> of the duodenum.

**Estimation of 5 Hydroxytryptamine** — The spectrophotofluorometric method of Udenfriend *et al* (1955) as modified by Wersbach (1961) was used for the determination of the 5 HT content in the duodenal pieces. The specimens were homogenized in 0.1 N HCl. From six saturated homogenate 5 HT was extracted into butanol and transferred with heptane into 0.1 N HCl. Concentrated HCl was added and the measurement was performed. The activation peak was at 296 nm and the fluorescence peak at 550 nm. 5 Hydroxytryptamine creatinine sulphate (Serotonin-creatinin sulfat Monohydrat Fluka AG) was used as a standard.

## STATISTICAL ANALYSIS

Student's *t* test was mainly used in the statistical analysis and in a special case an analysis of variance was also performed (Franko 1955b).

## RESULTS

### I HISTOCHEMICAL STUDIES

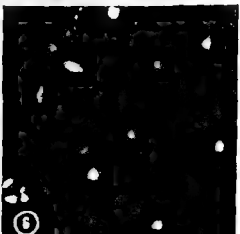
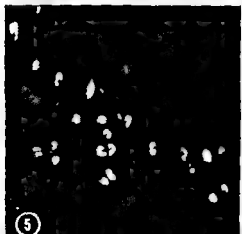
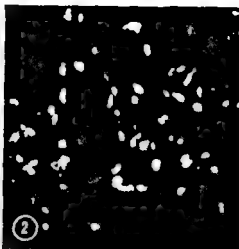
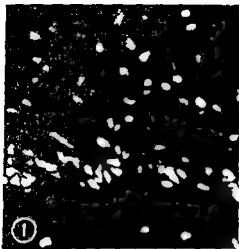
#### FORMALDEHYDE—INDUCED FLUORESCENCE

**General Observations** — In all the species investigated EC showed a strong formaldehyde induced fluorescence. No essential differences in the fluorescence intensity were observed between the different duodenal areas or the various species. The shape of the EC varied from round to triangular in the basal parts of the mucosa in the villi they were mainly columnar. Only in the rabbit the shape varied considerably in the villi where many strikingly narrow and long EC were seen. The EC were fairly similar in size in the different species. In the guinea pig they were however often a little larger and in the mouse rat and rabbit often smaller than in the other animals.

In all species the cytoplasm of the EC was filled with coarse strongly fluorescent granules. These granules were most numerous in the basal part of the EC where also the fluorescence was most intense. The granules were more easily distinguished in the other parts of the EC because of the weaker fluorescence. No essential difference in the size of the granules and in their abundance was noticed between various species in ultraviolet microscopy. In all the animals except the horse there were always some EC in which a narrow fluorescent strip of cytoplasm extended to the intestinal lumen. Such strips were very numerous in the sheep. The nuclei of the EC showed no fluorescence and they were seen as blackish spots in most EC. The nuclei were generally in the basal part of the cells.

The EC fluorescence was very stable. Even when the fluorescence of nerve fibres was diffuse indicating diffusion of catecholamines the EC remained sharply delineated and strongly fluorescent. If sections were immersed in water without deparaffinization the fluorescence of most EC did not disappear. On the covered object glass without mounting medium the distinct EC fluorescence lasted 6–12 months at room temperature and humidity.

**Sheep** — The fluorescence microscope field was dominated by a thin and dense band of EC (Fig. 1). Most of them were situated in the crypts of Lieberkuhn. In each crypt there were in general 5–20 EC, most of them side by side. Sometimes aggregations of 100–200 EC were seen. Only a few EC were found in the villi and in the submucosa. The fluorescence colour was a bright yellow with a faint nuance to brown and it did not weaken after a longer exposure to ultraviolet light.



**Cow** — All sections of the cow showed several fluorescent EC although the microscope field was dominated by green fluorescent dopamine cells (Fig 2). In each crypt of Lieberkuhn there were 4–12 EC and dense cell accumulations were sometimes seen in this part of the mucosa. Elsewhere in the mucosa only some isolated EC were observed and none of them in the submucosa. The EC showed a yellow fluorescence with a brownish nuance. The fluorescence intensity remained strong even after a long exposure to ultraviolet light.

**Pig** — The EC of the pig were seen as strongly fluorescent clumps in the ultraviolet microscope. In every Lieberkuhn's crypt there were 2–10 EC and sometimes aggregations of 20–30 cells were seen (Fig 3). In the villi and the submucosa only few EC were observed. The colour of the fluorescence in the EC granules was a bright yellow and its intensity remained unchanged after 1 hour's exposure to UV light.

**Horse** — Only a few fluorescent EC were seen in the microscope field (Fig 4). They were scattered quite evenly in the basal part of the mucosa. Rarely there were two EC side by side and no aggregations were seen. The thick clumpish villi contained very few EC and none of them were found in the submucosa. After a longer UV illumination the yellow fluorescence of the EC faded to some degree.

**Guinea Pig** — The incidence of fluorescent EC was largest in the basal part of the mucosa (Fig 5). In the crypts of Lieberkuhn there were 1–5 EC and seldom larger cell aggregations were seen. Isolated EC were noticed also in the submucosa and among the villous epithelial cells where their distribution was quite even. The fluorescent EC exhibited a yellow colour with a greenish nuance. Some decrease in the fluorescence intensity was observed after 1 hour's UV illumination.

**Mouse** — Only few EC were seen in the microscope field (Fig 6). Most of them were distributed quite evenly in the basal part of the mucosa and no cell aggregations were seen. Most crypts of Lieberkuhn contained no EC. The fluorescence was bright yellow in colour. After quite a short illumination its intensity weakened more clearly than that in the other species examined.

Formaldehyde induced fluorescence in the duodenum of mammals. The basal parts of the duodenum are seen in the lower areas of the figures.

*Fig 1* Sheep. Note the great density of EC in the basal part of the mucosa. Most of the fluorescent cells in the superficial part of the mucosa are dopamine cells. In the submucosa there are some strongly fluorescent dopamine cells.  $\times 160$ .

*Fig 2* Cow. Small EC aggregations are seen in the basal part of the mucosa. Most of the strongly fluorescent cells in the superficial part of the mucosa are polymorphic dopamine cells.  $\times 160$ .

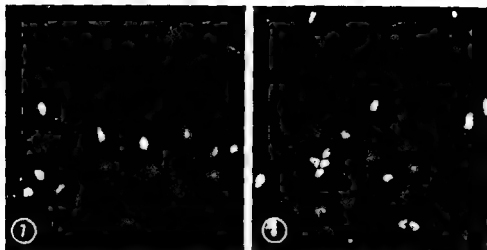
*Fig 3* Pig. The strongly fluorescent EC are diffusely distributed in the basal part of the mucosa.  $\times 160$ .

*Fig 4* Horse. Most of the few strongly fluorescent EC are located in the basal part of the mucosa.  $\times 160$ .

*Fig 5* Guinea pig. Small strongly fluorescent EC patterns are seen in the basal part of the mucosa. Most EC have non fluorescent nuclei.  $\times 160$ .

*Fig 6* Mouse. A few strongly fluorescent EC are seen in the basal and villous parts of the mucosa. Some strongly fluorescent small mast cells are present in the proximal part of the villi.  $\times 225$ .





Formaldehyde induced fluorescence in the duodenum of animals  $\times 160$

*Fig 7* Rat In the basal part of the mucosa only some strongly fluorescent EC are seen

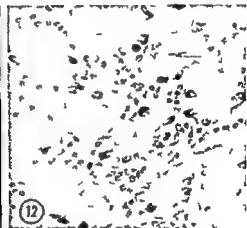
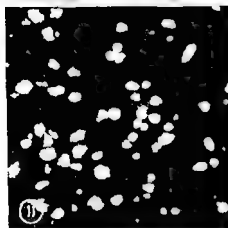
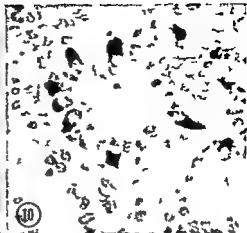
*Fig 8* Rabbit In the basal part of the mucosa there are small strongly fluorescent EC aggregations. Some degree of polymorphism of EC is seen

**Rat** — Also in the rat no EC accumulations were observed (*Fig 7*). Most EC were situated in the crypts of Lieberkuhn. One crypt seldom contained two cells. Some individual EC were observed among the epithelial cells of the villi. The colour of the fluorescence was a bright yellow. The intensity weakened quite quickly under ultraviolet light.

**Rabbit** — As in the other animals the largest EC patterns were in the basal part of the mucosa (*Fig 8*). However more numerous villous EC were seen in the rabbit than in the other animals studied. The distribution of the EC in the mucosa was quite even. No larger cell aggregations were seen. In the thick submucosa there were some separate EC. The bright yellow fluorescence colour faded gradually under ultraviolet action.

**Other Fluorescent Structures** — Few fluorescent mast cells were often seen in the duodenum of the pig, horse, guinea pig, mouse, rat and rabbit. Their yellow fluorescence was quite similar to that of the EC. In the pig, horse and rabbit these fluorescent mast cells were more often present in the submucosa. In the guinea pig, mouse and rat they were most numerous in the central areas of the villi. The mast cells were generally located between glandular elements around the blood vessels and were smaller and more irregular in shape than the EC. Therefore in spite of the similar yellow fluorescence these two cell types could be easily distinguished from each other.

In the duodenum of the sheep and cow there were numerous fluorescent dopamine cells with a greenish granular cytoplasm (*Figs 9–12*). They were distributed all over the mucosa and submucosa and some of them were also in the myenteric layers and subserosa, particularly around the blood vessels. In the cow they were especially numerous in the central part of the villi. The ratio of the EC and dopamine cells in the duodenum of the sheep was 1:2 and of the



*Fig 9* Formaldehyde-induced fluorescence in the cow duodenum. Strongly fluorescent EC and dopamine cells are seen in one crypt of Lieberkuhn.  $\times 320$

*Fig 10* The same section after the argyrophil reaction. Only the EC are specifically stained by the argyrophil reaction.  $\times 320$

*Fig 11* Formaldehyde induced fluorescence in the cow duodenum. Numerous strongly fluorescent EC and dopamine cells are seen.  $\times 160$

*Fig 12* The same section after the argentaffin reaction. Only the EC are specifically stained by the argentaffin reaction.  $\times 160$

cow 12—4 (five specimens were counted in each case). Because of the different fluorescence colours the EC and the dopamine cells were easily distinguished from each other even in a low magnification (100 $\times$ ).

A strong greenish fluorescence was seen in the submucous and myenteric nerve plexus of the duodenum. Delicate greenish fluorescent nerve fibres were often present in the mucosa and submucosa in the myenteric layers and in the subserosa, particularly around the smaller arteries and arterioles. This nerve fluorescence was an indication of a successful freeze-drying procedure. No connection was discerned between the fluorescent EC and nerve fibres.

In all species there were granular brownish yellow fluorescent cells located in the lamina propria of the intestinal villi and Payer's patches. Because of their location and different fluorescence properties these cells clearly differed from the EC. Their fluorescence was stable and it was also seen in the specimens not exposed to formaldehyde.

The duodenal mucous surface often contained a thin fluorescent layer of mucus varying in colour from yellow to red. In the intestinal lumen there was often fluorescent material of various colours. Both these types of fluorescence were mostly due to autofluorescence.

The background of the sections showed generally a very weak dirty green fluorescence.

A yellow fluorescence similar to that of the EC and the mast cells was not observed anywhere else in the duodenum.

## STAINING REACTIONS

**General Observations** — In each species the specifically coloured EC in the staining reactions were always situated in the same areas of the mucosa and submucosa as in the fluorescence reaction. The largest patterns of stained EC were seen in the basal part of the mucosa especially in the crypts of Lieberkuhn. Separate EC were observed in the villi and in the submucosa. In the muscular and subserous layers there were no positive EC. All the EC in each species stained strongly positive in the argyrophil reaction. In the other staining reactions the cytoplasmic colour intensity of EC varied considerably but no general difference was noticed between the villous and basal EC in the staining intensity. All the reactions were located in coarse granules in the cytoplasm of the EC and the granules were most numerous in the basal part of the cells. The shape and size of the stained EC exactly corresponded to those of the fluorescent EC. On the other hand fluorescent mast cells failed to exhibit positive silver reactions and also in the dopamine cells these reactions were very weak in comparison to those of EC (Figs 9—12). In the other staining reactions these cell types stained as the background.

**Argyrophil Reaction** — In all species the EC were stained a strongly deep blue or black and no essential difference in the colouring of the EC was observed between the species investigated. The EC were easily distinguishable from the other intestinal cells (Figs 13—19 and 56). Especially in the sheep, pig and rabbit there were often seen narrow and strongly coloured cytoplasmic strips extending to the intestinal lumen. Such strips were not observed in the horse and the mouse.

Argyrophil reaction in the duodenum of mammals  $\times 160$

Fig. 13 Sheep Numerous strongly stained EC are seen in the crypts of Lieberkuhn.

Fig. 14 Cow Numerous strongly stained EC are seen in the crypts of Lieberkuhn.

Fig. 15 Pig Some isolated strongly stained EC are seen in the crypts of Lieberkuhn.

Fig. 16 Horse A few strongly stained EC are seen in the crypts of Lieberkuhn.

Fig. 17 Mouse Some strongly stained EC are seen in the crypts of Lieberkuhn. The section taken near the pylorus.

Fig. 18 Rat Some strongly stained EC are seen in the crypts of Lieberkuhn.

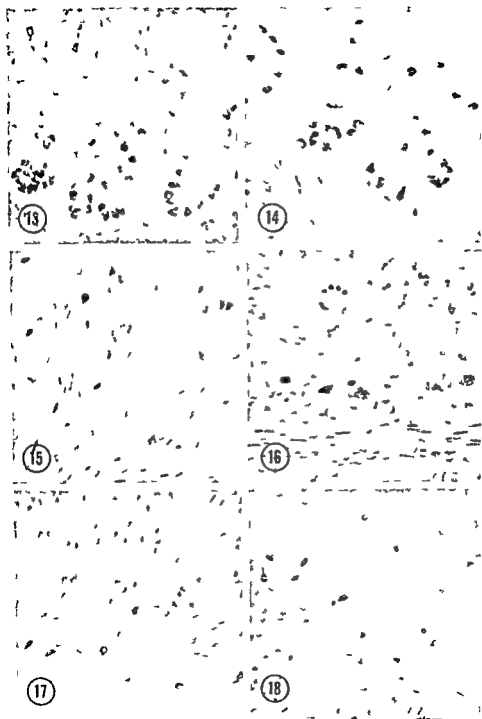




Fig 19 Argyrophil reaction in the rabbit duodenum. Some strongly stained I.C. are seen in the crypts of Lieberkuhn  $\times 160$

Fig 20 Argentaffin reaction in the rabbit duodenum. Some of the I.C. in the crypts of Lieberkuhn are more strongly stained  $\times 160$

The argyrophil reaction stained the cytoplasm of all the other cells very weakly although the staining time was long. On the other hand the nuclei of the other cells became moderately blue sometimes reddish. If the staining time was too long the whole section turned blackish because of the great density of nuclei.

The nerve plexus stained moderately blue in each species and was easily distinguished in the muscular layers and submucosa. In the mucosa delicate nerve fibres were seen but they were in no connection with I.C. Unspecific silver precipitates accumulated in almost every section. These stained clumps were easily distinguished from the stained tissues.

**Argentaffin Reaction** — The intensity of the argentaffin reaction in EC was strong in the sheep, cow, pig, horse and guinea pig, moderate in the rat and rabbit and weak in the mouse (Figs 20–26 and 66). The colour varied from black to light brown. In the pig and rabbit narrow cytoplasmic strips extended to the intestinal lumen. The nuclei were only slightly stained.

The neighbouring tissue was in general weakly stained. The colour varied in different nuances of brown. If the staining time was long, the whole section turned black. The nuclei of the other cells were stained a slightly deeper brown than

#### Argentaffin reaction in the duodenum of mammals $\times 160$

Fig 21 Sheep. Numerous strongly stained I.C. are seen in the crypts of Lieberkuhn.

Fig 22 Cow. Numerous strongly stained I.C. are seen in the crypts of Lieberkuhn.

Fig 23 Pig. Some strongly stained I.C. are seen in the crypts of Lieberkuhn.

Fig 24 Horse. A few strongly stained I.C. are seen in the crypts of Lieberkuhn.

Fig 25 Mouse. Most of the I.C. are weakly stained. The section was taken close to the pylorus.

Fig 26 Rat. Some of the I.C. are more strongly stained. The section was taken near the pylorus.



**Ferric Ferricyanide Reaction** — The staining intensity of the EC varied considerably between the different species. It was very strong in the pig and guinea pig, moderate in the cow and rabbit, and weak in the sheep, horse and rat (Figs 27—29 and 70). In the mouse only a few EC were positive. In the duodenum of the mouse and rat the stained EC were most numerous near the pylorus where also the reaction was more intense than in the other parts of the duodenum. In all species the colour of the coarse EC granules varied from deep blue to light green and the narrow apical parts of the EC as well as the nuclei were almost colourless.

All reactive EC were easily discerned among the other cells. The background was in general light green and its colour depended on the staining time. After a longer reaction time the sections became a deep blue. The nuclei of the other cells exhibited a more intense reaction than their cytoplasm.

**Diazo Coupling Reaction** — This reaction was strong in the pig and guinea pig, weak in the sheep, cow and rabbit but just visible in the horse, mouse and rat (Figs 30—32 and 74). In the mouse and rat reactive EC were mostly seen near the pylorus where also the reaction intensity was a little stronger. The colour of the EC varied from brownish red to light yellow. The apical parts of the EC were almost colourless.

In general the background was stained light yellow but after a longer staining it turned a deep brown. The strongly coloured EC were easily distinguishable. No essential colour difference was seen between the cytoplasm and the nuclei in the other cells. In the blood vessels and in the central part of the villi there were often small strongly stained cells which were easily distinguished from the EC.

**Indophenol Reaction** — The indophenol reaction was negative in the EC of the horse, mouse and rat (Fig. 35). Also in the cow and rabbit the reactive EC were only just visible. In the sheep (Fig. 33) the reaction intensity was moderate whereas in the pig (Fig. 34) and guinea pig (Fig. 78) it was strong. The colour of the reaction was grey or blackish. The apical parts of the EC, cytoplasm were almost colourless in each species. The nuclei of the EC stained very weakly.

The background of the sections remained almost colourless and the stained EC were easily discerned as dark points in the sections. After several hours staining also the background turned a dirty grey and especially some cells in the blood vessels were stained blackish. The nucleus and cytoplasm of the other cells were similarly intensively stained.

## AUTOFLUORESCENCE

The EC showed no autofluorescence in the species investigated.

*The results obtained with the different reactions are summarized in Table I.* The mean fluorescence and staining intensity of the EC in the various species was labelled as follows: negative — just visible + weak ++ moderate --- strong ++++. All the reactions were strongly positive in the EC of the guinea pig. Therefore the average intensity in this animal was recorded in each reaction as ---. The intensity of the different reactions in the EC of the other species was compared with that of the guinea pig.





The number of fluorescent EC was indicated by the sign ○○○○. In the different staining reactions the number of positive EC was compared with that of the fluorescent EC and the result was designated as follows: no positive FC—few positive EC ○ some positive EC ○■ numerous positive EC ○○○ is many as fluorescent EC ○○○○.

Serial sections taken from each specimen were used for the calculation of these mean amounts of EC and their average reaction intensity (magnification 200 or 400 ×). These calculations are summarized in Table 1.

It is quite clear that there is a correlation between the number of EC shown by the various methods and the intensity of these reactions in the EC, i.e. that methods giving a weak reaction also make fewer EC visible.

## DISCUSSION

All the species investigated showed numerous LC in the duodenum. This observation is in agreement with many earlier studies (Møller 1899, Tang 1922, Hamperl 1925, Kull 1925, Cordier 1926, Toro 1929, Eros 1930, Tehver 1930, Clara 1933a, Jacobson 1939).

### *Silver Reactions*

In each species all the EC stained strongly in the argyrophil reaction. On the other hand, in the argentaffin reaction the staining intensity of the LC varied in different species. In the sheep, cow, horse and guinea pig the FC exhibited an intense argentaffin reaction, whereas the reaction intensity was weaker in the other animals. Besides in the horse, mouse, rat and rabbit the LC in the argentaffin reaction were less numerous than those in the argyrophil reaction. This kind of distinct difference in the number of the argyrophil and argentaffin FC has been earlier observed by Dawson (1945, 1948) in the stomach of several laboratory animals by Hellweg (1952), Hamperl (1952), Hardmeier and Hedinger (1965) and Singh (1965a) in the human gastrointestinal tract, as well as by Eder *et al.* (1959) and Prellwitz (1959) in the duodenum of the guinea pig. According to all these investigations the argyrophil EC were more numerous than the argentaffin EC.

In the present study it was, however, observed that in the sheep, cow, pig and guinea pig the argentaffin EC were as numerous as the argyrophil FC. This observation is obviously due to the fact that the tissue pieces were taken close to the pylorus, for it was stated by Tehver (1930), Clara (1933a) and Singh (1965a) that in the proximal part of the duodenum the argentaffin EC were more numerous and more easily demonstrable than in the other duodenal parts. Obviously the FC near the pylorus contain a lower turnover rate of 5-HT and much more substances which have argentaffin properties than the FC in the other duodenal areas.

The great difference between the argyrophil and argentaffin reactions in demonstrating the FC must be caused principally by dissimilar properties in the EC granules or the argyrophil reaction is a much more sensitive one. The argyrophil FC may be the precursors of the argentaffin FC as postulated by Frispmann (1937) or they may be in another physiological state. Because of the lack of chemical specificity of the argyrophil reaction, the substance causing this reaction

in the EC is entirely obscure but it is of interest that it remains in the enterochromaffin granules even after alcohol fixation (Eder *et al* 1962) when the other staining reactions are negative. However the argyrophil reaction is more reliable in demonstrating the EC than the more specific argentaffin reaction.

### *Other Staining Reactions*

Great differences were observed between the various reactions with respect to the staining ability of the EC. The silver reactions were more effective for demonstrating the EC than either the ferric ferricyanide or the diazo coupling reaction. The indophenol reaction was distinctly less effective than all the other reactions. However all the reactions were strongly positive in the EC of the pig and guinea pig. In the other species the ferric ferricyanide and diazo coupling reactions stained the EC less effectively and the indophenol reaction was entirely negative in the EC of the horse, mouse and rat.

The results obtained by earlier investigators are equally variable. The argentaffin, ferric ferricyanide and diazo coupling reactions were demonstrated to be equally effective in the staining of the EC in the human appendix (Laskey and Greco 1948), in the duodenum of the guinea pig (Prellwitz 1959) and in the human large bowel (Hardmeier and Hedinger 1965). On the other hand Eder *et al* (1959) demonstrated that in the duodenum of the guinea pig the argentaffin reaction stained about twice as many EC as the ferric ferricyanide, diazo coupling and indophenol reactions. Only some of the EC that were positive in the ferric ferricyanide reaction were also diazo positive according to Lillie *et al* (1961). However the results obtained in the present study showed that the reaction intensity and the EC count are in a clear correlation to each other in mammalian species.

It has been in general assumed that all the staining reactions with the exception of the argyrophil reaction are caused by the same substance in the EC, i.e. a condensation product of 5 HT and formaldehyde (Barter and Pearse 1955, Pearse 1960). The intensities of the staining reactions show a direct correlation with the 5 HT concentration as was demonstrated by Benditt and Wong (1957) in gelatine models. It seems therefore that the 5 HT content of the EC determines the reaction intensity in great degree. The influence of the precursors of 5 HT and other chemical substances on the staining properties of the EC is obscure. Also the granular structure and the mechanism by which the amines are bound to the granules may be different in various species and cause staining differences. Besides the physiological state of the animals, the secretory and metabolic activity of the EC, the anatomical point of the tissue pieces as well as the staining method used certainly have an influence on the colour intensity of the EC.

### *Fluorescence Reaction*

The fluorescence reaction was golden yellow in intestinal EC fixed in formaldehyde solutions (Eros 1932). The sensitivity of the reaction in this method was equivalent to that of the staining reactions (Benditt and Wong 1957).

In the present study the EC exhibited a very strong formaldehyde induced fluorescence in all the species investigated. The fluorescence colour was bright

yellow. The tendencies towards brown or green in the EC of the sheep, cow and guinea pig obviously were due to different concentrations of amines or other substances disturbing the fluorescence.

According to Falck and Owman (1965) the yellow fluorescence colour is specific for certain tryptamine derivatives. However, subjective estimation of the fluorescence colour is subject to considerable errors and therefore it is better not to depend too much on the colour characteristics. Falck and Owman observed that the 5-HT fluorescence disappeared more rapidly than the catecholamine fluorescence. In the present study, no such essential weakening of the LC fluorescence was observed even after a long exposure to UV light and the speed of fluorescence fading is not a good criterion for 5-HT.

In view of the contact between the narrow but always strongly yellow fluorescent cytoplasmic strips and the intestinal lumen, it seems likely that the EC also have an exocrine secretory function, as has been suggested by Erspamer (1961). Besides the coarse granularity of the EC cytoplasm shows a granular form of storage of the biologically active amines.

In the duodenum of different species a yellow fluorescence of this kind was observed only in the EC and mast cells. The nerve plexus exhibited only a greenish fluorescence. Therefore it is quite possible that most, if not all, of the intestinal 5-HT is concentrated in these cells *in vivo*. With the aid of the fluorescence reaction it is however impossible to determine whether there are other fluorescent tryptamine derivatives in the EC.

It has been demonstrated that the mast cells of the mouse and rat contain considerable amounts of 5-HT, whereas this amine has been considered to be lacking from the mast cells of other species (Benditt *et al.* 1955; Parrat and West 1957; Falck 1962). In the present study, mast cells with a strong yellow fluorescence were found in the duodenum of the pig, horse, guinea pig, mouse, rat and rabbit. The intense yellow fluorescence suggests that tryptamine derivatives, obviously 5-HT, are a normal component of the mast cells. The small 5-HT content of the mast cells was probably overlooked in the earlier studies because the methods used were not adequately sensitive.

Falck *et al.* (1959) found in the gut of ungulates cells which presumably contained dopamine. Coupland and Heath (1961) regarded these cells as typical mast cells. In the present study, such cells were found in the sheep and cow duodenum. The fluorescence of these cells was green and distinctly differed from the yellow fluorescence of the LC and it was probably due to dopamine. Because very numerous dopamine cells were seen only in the sheep and cow, in contrast to the very few mast cells in all the other species, it is likely that their physiological function is different from that of the mast cells. It is moreover open to question whether the dopamine cells are true mast cells, as claimed by Coupland and Heath (1961). However, both cells were not specifically stained in the various staining reactions.

## II CONSECUTIVE DEMONSTRATION OF FORMALDEHYDE-INDUCED FLUORESCENCE AND STAINING REACTIONS IN ENTEROCHROMAFFIN CELLS

### RESULTS

After microphotographs were taken from the fluorescent EC the same sections were stained with the specific reactions and rephotographed

**Argyrophil Reaction** — In all the eight species all the fluorescent EC exhibited a strongly positive silver impregnation reaction (Figs 36 and 37)

**Argentaffin Reaction** — In the sheep cow pig and guinea pig (Figs 38 and 39) all the fluorescent EC were also argentaffin. In the horse and rabbit most fluorescent EC were positive also in the argentaffin reaction whereas in the mouse and rat a few fluorescent EC were argentaffin

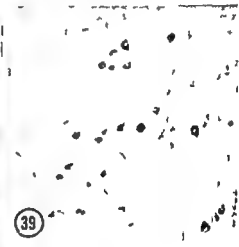
**Ferric Ferricyanide Reaction** — The comparison was made only in the pig and guinea pig. In both animals all the fluorescent EC showed a positive ferric ferricyanide reaction (Figs 40 and 41)

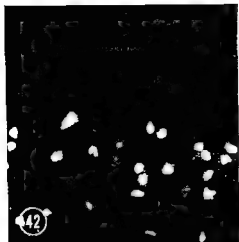
**Diazo Coupling Reaction** — The correlation was studied only in the pig and guinea pig. All the fluorescent EC were stained by the diazo coupling reaction. However some of the fluorescent EC were less intensively stained (Figs 42 and 43)

**Indophenol Reaction** — All the fluorescent EC of the pig and guinea pig duodenum were stained by this reaction (Figs 44 and 45). However the staining of some EC was weak particularly in the pig

### DISCUSSION

Whether the same EC are reactive towards all different staining reactions has been hardly examined. The earlier observations were made only in the adjacent sections which is a method of questionable value. Singh (1963, 1964a) used double staining. He first stained the EC of the foetal and adult human gastrointestinal tract using either the argentaffin, ferric ferricyanide or diazo coupling reaction. Thereafter the sections were stained by the argyrophil method. Singh demonstrated that all the EC positive in the argentaffin, ferric ferricyanide and diazo coupling reactions were also stained in the argyrophil reaction even though the argyrophil EC were more numerous than the positive EC in the other reactions. However it is not clear how much the first reaction affected the second one possibly causing an erroneous positive reaction.





Consecutive demonstration of formaldehyde induced fluorescence and staining reactions in EC  
 $\times 160$

*Fig 42* Formaldehyde induced fluorescence in the guinea pig duodenum

*Fig 43* The same section after the diazo coupling reaction All the fluorescent EC are specifically stained also in the diazo coupling reaction

*Fig 44* Formaldehyde induced fluorescence in the guinea pig duodenum

*Fig 45* The same section after the indophenol reaction All the fluorescent EC are specifically stained also in the indophenol reaction. Some fluorescent EC are weakly stained

Consecutive demonstration of formaldehyde induced fluorescence and staining reactions in EC  
 $\times 160$

*Fig 36* Formaldehyde induced fluorescence in the guinea pig duodenum

*Fig 37* The same section after the argyrophil reaction All the fluorescent EC are specifically stained also in the argyrophil reaction

*Fig 38* Formaldehyde induced fluorescence in the guinea pig duodenum

*Fig 39* The same section after the argentaffin reaction All the fluorescent EC are specifically stained also in the argentaffin reaction

*Fig 40* Formaldehyde induced fluorescence in the guinea pig duodenum

*Fig 41* The same section after the ferric ferricyanide reaction All the fluorescent EC are specifically stained also in the ferric ferricyanide reaction

The fluorescence reaction has not previously been correlated with the staining reactions although it offers unique possibilities for such a comparison since the second reaction can be applied to an unstained section.

In the present study it was undeniably demonstrated that the EC in all the mammals investigated were composed of morphologically similar fluorescent cells. It was further noted that all the fluorescent EC were positive also in the argyrophil reaction. In the pig and guinea pig all the other staining reactions also were positive in the same EC as the fluorescence reaction as was already indicated by cell count calculations in serial paraffin sections. Only some of the fluorescent EC exhibited a positive argentaffin reaction in the horse, mouse, rat and rabbit whereas in the sheep and cow they all were also argentaffin. The stainings performed after the fluorescence reaction excluded the possibility that there were duodenal EC which were positive only in the argentaffin reaction without argyrophil properties as postulated by Hellweg (1952) and Hamperl (1952). On the other hand, in all the species investigated there were no stained EC without a yellow fluorescence.

The yellow colour of the fluorescence suggests that the enterochromaffin system at least in mammals is composed of cells all containing similar chemical substances. The stages of the pre-enterochromaffin non-argyrophil and empty EC suggested by Erspamer (1937) were not demonstrable in the present study with the highly sensitive fluorescence reaction which observation abolishes Erspamer's theory at least in adult mammals. It was also impossible to find any intermediate cell forms between the EC and intestinal epithelial cells from which the EC are obviously derived (Cordier 1926, Friedmann 1934). Therefore it is probable that in the adult mammals the enterochromaffin system is composed of one cell type only the principal properties of which are the yellow formaldehyde induced fluorescence and the strong argyrophilia.

### III ENTEROCHROMAFFIN CELLS AND 5 HYDROXYTRYPTAMINE IN THE DUODENUM

#### CORRELATION BETWEEN THE NUMBER OF ENTEROCHROMAFFIN CELLS AND THE $\gamma$ HYDROXYTRYPTAMINE CONCENTRATION

##### *Material*

A total number of 109 animals were studied. The EC were counted and the 5-HT content was determined in the duodenal specimens of 23 sheep 14 cows 17 pigs 12 horses 10 guinea pigs 16 mice 11 rats and 6 rabbits. If the freeze drying procedure was not successful, the specimens were rejected.

##### *Results*

The diameter of the fluorescent EC was 10.7  $\mu$  in the sheep 10.3 in the cow 11.1 in the pig 10.8 in the horse 11.7 in the guinea pig 9.5 in the mouse 9.7 in the rat and 9.9 in the rabbit. The smallest vertical height of the fluorescent structures which could be identified as EC was 3  $\mu$ .

**Enterochromaffin Cells** — The largest mean value of the EC (7 040 EC/mm<sup>2</sup> tissue) was observed in the duodenum of the guinea pig. Also in the sheep and rabbit the EC were remarkably numerous. Their mean values (6 260/mm<sup>2</sup> and 4 940/mm<sup>2</sup>) however differed significantly ( $P < 0.05$  and  $P < 0.001$  respectively) from the mean value of the guinea pig and from each other ( $P < 0.01$ ). The mean EC counts of the rat (3 340/mm<sup>2</sup>) cow (2 970/mm<sup>2</sup>) pig (2 450/mm<sup>2</sup>) and mouse (2 170/mm<sup>2</sup>) were of the same size class. The EC count of the horse (850/mm<sup>2</sup>) differed considerably ( $P < 0.001$ ) from all the other animals. The standard deviations varied between 9.8 % and 25.4 % (Fig. 46).

**5 Hydroxytryptamine** — The highest 5-HT concentrations were found in the sheep (the mean value 24.9  $\mu$ g/g tissue) and the guinea pig (19.2  $\mu$ g). The 5-HT contents were also considerably high and almost equally large in the cow (13.9  $\mu$ g) the pig (13.1  $\mu$ g) and the rabbit (11.0  $\mu$ g). All the other animals showed distinctly smaller 5-HT concentrations. However the mouse (6.3  $\mu$ g) differed significantly ( $P < 0.001$ ) from the horse (3.2  $\mu$ g) and the rat (3.0  $\mu$ g). The standard deviations varied from 11.9 % to 49.4 % (Fig. 46).

**Correlation between the Enterochromaffin Cells and 5 Hydroxytryptamine** — The relationship is represented in Fig. 47 with a corresponding regression line defined from the mean values for each species. The regression equation is  $y = 1.25 + 0.0028x$  with a correlation coefficient



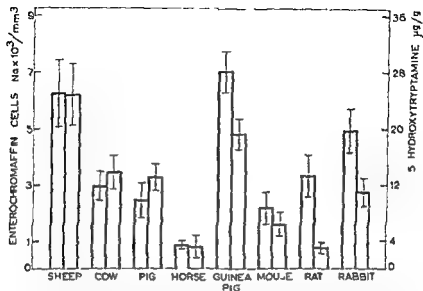


Fig 46 The number of EC and the 5-HIT concentration in the duodenum of mammals. Ordinate left EC No  $\times 10^3/\text{mm}^3$  duodenal tissue. Ordinate right 5-HIT concentration  $\mu\text{g/g}$  duodenal tissue. Across animal species EC hatched 5-HIT unhatched. Means and standard deviations.

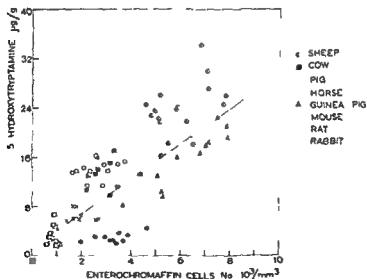


Fig 47 Regression line demonstrating the relationship between the EC number and the 5-HIT concentration in the duodenum of mammals. Ordinate left 5-HIT concentration  $\mu\text{g/g}$  duodenal tissue. Abscissa EC No  $\times 10^3/\text{mm}^3$  duodenal tissue.  $y = 1.25 + 0.0028x$  correlation coef  $r = 0.79$  which is highly significant ( $P < 0.001$ ,  $n = 109$ ).

cient  $r = 0.79$  which is highly significant ( $P < 0.001$   $n = 109$ ). The individual points of the sheep, cow and pig are above the regression line whereas those of the rat and rabbit are all below it. Only the points of the horse, guinea pig and mouse are distinctly on both sides of the regression line.

### MEAN 5-HYDROXYTRYPTAMINE CONTENT OF THE ENTEROCHROMAFFIN CELLS

It was assumed that all the 5-HT in the duodenum located in the EC. The calculation of the mean 5-HT content in one EC was based on the EC counts, 5-HT concentrations and specific gravities of the duodenum and was made by dividing the 5-HT content by the EC number in the same tissue volume.

The specific gravity of the duodenal tissue was 1.058 in the sheep, 1.056 in the cow, guinea pig and rabbit and 1.055 in the pig, horse, mouse and rat. The specific gravity was determined in sucrose solutions. The table published by Geigy (1960) was used.

The EC of the pig, cow, sheep and horse showed the largest mean 5-HT content in one EC (Fig. 48). The mean values in these species varied in the range  $5.9 - 4.1 \times 10^{-6} \mu\text{g}$  5-HT. In the mouse, guinea pig and rabbit the mean values were smaller and varied from  $3.3$  to  $2.4 \times 10^{-6} \mu\text{g}$ . In comparison to all the other animals, the 5-HT concentration was very low in the EC of the rat ( $1.2 \times 10^{-6} \mu\text{g}$ ) and differed very significantly ( $P < 0.001$ ) from them. The standard deviations varied between 12.3% and 47.1% and were considerably high in the horse and mouse.

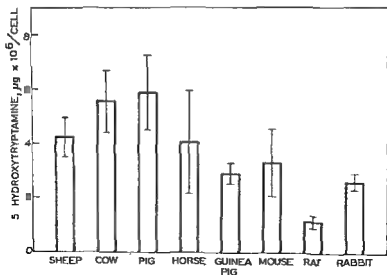


Fig. 48. The mean content of 5-HT in the EC of mammals. Ordinate: 5-HT  $\mu\text{g} \times 10^{-6}/\text{cell}$ . Abscissa: animal species. Means and standard deviations.

## DISTRIBUTION OF THE ENTEROCHROMAFFIN CELLS AND 5-HYDROXYTRYPTAMINE IN THE DIFFERENT DUODENAL LAYERS

### Material

Thirteen cows were used in the experiment because the duodenal wall was easily separated into two layers only in this animal. One of these layers consisted of the mucosa and submucosa and the other of the muscularis interna, muscularis externa and serosa layers. The ratio between the volumes of the separated layers was 1:00:1:01. With the aid of the usual haematoxylin-eosin staining it was confirmed that the separated layers comprised only the components stated.

### Results

The EC were observed neither in the muscularis externa or interna nor in the serosa or subserosa. The mean 5-HT content in these layers together was  $1.2 \mu\text{g/g}$  tissue. On the other hand, in the united mucous-submucous layer the mean EC count was  $5,900 \text{ EC/mm}^2$  tissue and correspondingly also the mean 5-HT concentration in this layer was considerably high ( $22.9 \mu\text{g/g}$  tissue). The values for the mucous-submucous layer were about twice as high as for the whole duodenal wall (Fig. 49).

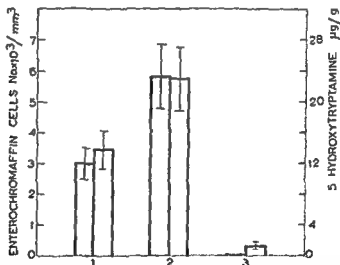


Fig. 49 The number of EC and the 5-HT concentration in the different layers of the cow duodenum. Ordinate left: EC No.  $\times 10^3 / \text{mm}^2$  duodenal tissue. Ordinate right: 5-HT concentration  $\mu\text{g/g}$  duodenal tissue. Group 1: the whole duodenal wall; group 2: the united mucous and submucous layers; group 3: the united internal muscular, external muscular and serous layers. EC: hatched; 5-HT: unhatched. Means and standard deviations.

## ENTEROCHROMAFFIN CELLS AND 5-HYDROXYTRYPTAMINE IN THE DEVELOPING DUODENUM

### Material

A total number of 54 rats and 9 guinea pigs were studied. The rats were 1–2 hours (8 rats), 4 days (9), 8 days (5), 12 days (5), 16 days (11), 24 days (5), 32 days (5), and 56 days (6) of age.

The nine guinea pigs were studied immediately after birth. The EC counts and the 5-HT concentrations in the duodenum were determined separately for each age group.

### Results

**Rat** — Strongly fluorescent yellow EC were seen at all ages of the rat. No difference in the fluorescence intensity of the EC was observed between the various ages. The size and shape of the EC in the newborn rat were quite similar to those in the older animals. However, it was noticed that the villous EC were proportionally more numerous in the younger than in the older animals. Few yellow fluorescent mast cells were seen, especially in the lamina propria of the villi. This kind of yellow fluorescence was not observed in the other tissue components.

The diameter of the fluorescent EC determined for the Floderus correction was  $9.9 \mu$  in the newborn rat,  $10.0$  in the 4 day old,  $9.8$  in the 8 day old,  $9.6$  in the 12 day old,  $9.7$  in the 16 day old,  $10.0$  in the 24-day old and in the 32 day old, and  $9.7$  in the 56 day old rat.

The EC count was significantly higher in the younger than in the older rats (Fig. 50). Immediately after birth and at the age of 4 days great individual variations were observed in the number of EC. The mean values for these ages were  $5260$  (EC/mm<sup>3</sup> tissue) and  $6570$ /mm<sup>3</sup>, the standard deviations  $27.2\%$  and  $16.9\%$  respectively. Thereafter a clear decrease was observed. A significant increase in the EC count was observed at the age of 16 days ( $P < 0.001$ ). A continual decrease in the EC count was noticed at the age of 24 and 32 days when the adult level was reached.

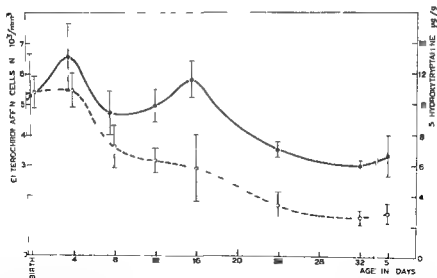


Fig. 50 The number of EC and the 5-HT concentration in the developing rat duodenum. Ordinate left: EC No.  $\times 10^3$ /mm<sup>3</sup> duodenal tissue. Ordinate right: 5-HT concentration  $\mu$ g/g duodenal tissue. Abscissa: age in days. EC marked — — — — — 5-HT marked — — — — —. Each point is a mean value  $\pm$  standard deviation.

A similar decrease was observed in the 5 HT concentration. It was at its highest in the newborn and 4-day old rats the mean values being 10.8 and 10.9 ( $\mu\text{g}$  5 HT/g tissue). Thereafter a continual decrease was observed and the adult level was reached between the ages of 24 and 56 days (Fig. 50).

**Guinea Pig** — In the duodenum of the guinea pig numerous strongly fluorescent yellow EC were observed immediately after birth. They were mainly situated in the basal part of the mucosa. Some separate EC were also found in the villi. No difference in the size and shape or the fluorescence intensity of the EC was noticed between the newborn and adult guinea pigs. Except for few fluorescent mast cells no other yellow fluorescent components were seen.

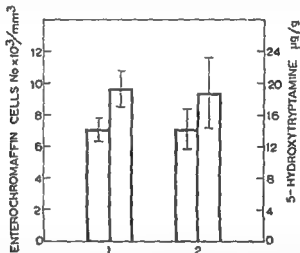


Fig. 51 The number of EC and the 5 HT concentration in the duodenum of the guinea pig. Ordinate left: EC No.  $\times 10^3/\text{mm}^3$  duodenal tissue. Ordinate right: 5 HT concentration  $\mu\text{g/g}$  duodenal tissue. Group 1: adult guinea pigs; group 2: newborn guinea pigs. EC: hatched; 5-HT: unhatched. Means and standard deviations.

No significant difference was noticed in the EC diameter between the newborn and adult guinea pigs, the mean values being 11.3 and 11.7  $\mu$  respectively.

The relationship between the EC count and the 5 HT concentration in the newborn and the adult guinea pig is seen in Fig. 51. The mean values of EC (7.090 EC/ $\text{mm}^3$  tissue) and 5 HT (19.4  $\mu\text{g/g}$  tissue) in the newborn guinea pig were quite similar to those in the adult, although the standard deviations were larger in the newborn animals.

## DISCUSSION

### *Enterochromaffin Cells*

The number of EC has been expressed in terms per section of stated size (Tehver 1930, Hoeschen 1937, Klemm 1937, Vetter 1937, 1938, Munch 1939), per visual field of stated size (Benditt and Wong 1957, Sullivan 1960, Funk *et al* 1966a, Funk *et al* 1966b), per one or several intestinal crypts (Verity *et al* 1962, Deschner 1965, Funk *et al* 1966a, Funk *et al* 1966b), per square millimeter of gut surface, per square millimeter of muscularis mucosae or per square millimeter

meter of basement membrane (Singh 1965b) With the aid of these methods however it is very difficult to make precise quantitative comparisons between the EC counts in different species or different parts of the intestinal tract because there are considerable variations in the intestinal structure and proportionate share of intestinal layers in the different species I have not been able to find any previous study in which the number of EC has been expressed in terms of volume of intestinal tissue

All the EC counts in the present study were expressed per unit volume of the whole intestinal wall which facilitates also the comparison of the EC density and the 5 HT concentration in the duodenum Owing to the thinness of the sections (5 micra) a great number of the counted EC in the sections were cell fragments and the primary results clearly exceeded the true values Therefore it was necessary to correct the results according to the Floderus formula (Eranko 1955b) For the use of this formula the particles must be of spherical form and randomly distributed in the tissue The counts determined in the present study are consequently approximate because the precise determination of the diameter of the EC was difficult in view of the mainly columnar cell shape In general the EC diameter was of the same size class in the different species and the various age groups Only the pig and guinea pig differed clearly from the other animals

The argentaffin reaction used in all calculations in the literature is less suitable for the demonstration of the EC in some animal species and some parts of the alimentary canal as was shown above in the present study Therefore only the formaldehyde induced fluorescence reaction was used for the determination of the EC counts

All the adult animals showed numerous fluorescent EC in their duodenum The highest EC counts were found in the guinea pig (7 040 EC/mm<sup>3</sup> duodenal tissue) and in the sheep Considerably lower numbers of EC were observed in the rabbit whereas in the rat cow pig and mouse the EC levels were of the same order of magnitude The small EC density of the horse (850/mm<sup>3</sup>) differed significantly ( $P < 0.001$ ) from that of all the other animals These observations lend further support to the investigations made by Tang (1922) Kull (1925) Cordier (1926) and Tehver (1930) According to their studies the EC were most numerous in the duodenum of the pig guinea pig and sheep and moderately numerous in the cow and rabbit while in the horse a few EC were found However according to Kull (1925) and Cordier (1926) the EC were very few in the mouse and rat and it was reported by Hoeschen (1937) and Munch (1939) that they were 5—8 times as numerous in the proximal part of the duodenum of the guinea pig as of the rat The inconsistency of these results is probably due to the inadequacy of the argentaffin method to stain all the EC in the mouse and the rat When the formaldehyde induced fluorescence reaction is used as in the present study the variations are not so great

In the present study all the EC counts of the domestic animals and rabbit were made from tissue pieces taken adjacent to the pylorus whereas in the guinea pig mouse and rat the tissue pieces were cut from the middle of the first third of the duodenum This proximity of the pylorus makes the EC counts considerably high as already shown by Hoeschen (1937) and Munch (1939) using the argentaffin reaction in the guinea pig and rat In the sheep cow pig horse and rabbit the proportion of the muscular and submucous layers in the intestinal

wall is significantly larger than in the other animals. These two inner layers compensate for the large species differences in the EC patterns seen solely in the mucosa when defining the EC count for the whole duodenal wall.

Schumann (1939) counted the EC by the section technique and found more numerous duodenal EC in the adult than in the newborn guinea pig. However, this kind of difference was not observed in the present study. Besides a considerably higher EC count was noted in the duodenum of the newborn (5 260 EC/mm<sup>2</sup> tissue in mean) than in the adult rat (3 340/mm<sup>2</sup>). During development the EC count decreased continuously and the adult level was reached at the age of 24–32 days. A very significant peak in the EC count was observed in the 16 day old rats which obviously indicates alterations at that age in the proportionate amounts of duodenal wall components. This kind of difference between newborn and adult animals in the EC counts was also observed by Tehver (1930) in the duodenum of the calf and the foal.

### *5 Hydroxytryptamine*

The 5 HT concentrations obtained in the earlier investigations vary considerably according to the animal species, the physiological state of the animals, the anatomical part of the intestinal tissue and the extraction and assay methods. The concentration of 5 HT (in µg/g tissue) in the small intestine has been reported in the sheep in the range 2.00–4.10, in the calf 3.00–3.60, in the pig 0.50–4.00, in the horse 0.32–0.72, in the guinea pig 3.4–14.4, in the mouse 1.0–1.6, in the rat 1.2–6.5 and in the rabbit 3.3–10.7 (Erspamer 1953, 1954, 1961; Faustini 1955; Weissbach *et al.* 1958; West 1958; Bertaccini 1959; Toh 1960; Tissari 1966).

In the present study the values obtained in the duodenum were considerably higher. The highest values were observed in the sheep (mean value 24.9 µg 5 HT/g tissue) and the guinea pig. In the cow, pig, rabbit and mouse the mean values were significantly lower and in the horse (3.2 µg) and the rat (3.0 µg) the lowest of all the animals studied. Erspamer (1953) and Faustini (1955) also demonstrated that in several animals the proximal parts of the small intestine showed considerably higher concentrations of 5 HT than the distal areas. Therefore the high values of the present study are not surprising.

In the newborn guinea pig the 5 HT concentration in the duodenum was quite similar to that of the adult guinea pig. This supports the observations made by Tissari (1966). It is therefore amazing that the duodenal 5 HT concentration was 160% higher in the newborn than in the adult rat. Such a difference has not been observed earlier. On the contrary, Bertaccini (1958) reported that the 5 HT content increased during development in the large intestine of the rat. It is probable that the decrease of 5 HT observed in the rat duodenum during growth is caused by the commencement of intestinal function (Bulbring and Crema 1959) or by an increased intraluminal pressure (Cole *et al.* 1961).

### *Correlation between the Enterochromaffin Cells and 5 Hydroxytryptamine*

By means of the fluorescence method it is possible to determine precisely the EC counts in the intestinal tissue. The mast cells which had a similar yellow fluorescence as the EC were easily differentiated by their morphology and location.

On the other hand the spectrophotofluorometric method used for the 5 HT determination is specific for all the 5 hydroxyindoles (Bowman *et al* 1955 Weissbach 1961) among which 5 HT is the most important one in the animal tissue. The kind of 5 HT precursors and derivatives in the EC is still obscure. Obviously their amount in the animal tissues is so low that they have no signification in the EC count and the 5 HT content determinations (Erspamer 1961). It has been pointed out that only in the mouse and the rat the mast cells contain detectable amounts of 5 HT (Benditt *et al* 1955 Parrat and West 1957 Coupland and Riley 1960). According to Keller (1957) the mast cells of the peritoneal cavity in the rat contain  $0.346-0.462 \times 10^{-6} \mu\text{g}$  5 HT/cell. In the present study the mean 5 HT content in one EC of the rat was about 3-4 times as high ( $1.2 \times 10^{-6} \mu\text{g}$ ). Owing to the small number of mast cells and their low 5 HT content it is obvious that the total amount of 5 HT located in these cells is negligible in comparison to that of the EC. Besides the present study showed that only traces of 5 HT could be found in the muscular and serous layers of the cow duodenum as earlier reported by Murray and Wylle (1964) on the human stomach. The 5 HT amount present in the platelets and in the blood plasma remaining in the duodenum is very small in comparison to that of the EC (Erspamer 1961).

In the investigations reported in the literature the EC count and the 5 HT content were determined separately only Verity *et al* (1962) used adjacent tissue pieces of the human large bowel. However because the EC count and the 5 HT concentration greatly depend on the anatomical and environmental conditions it is necessary to make the determinations from adjacent tissue pieces in order to obtain reliable correlation comparisons.

In all the adult mammals investigated there was a highly significant ( $P < 0.001$ ) correlation between the EC count and the 5 HT content in the duodenum. In the sheep, cow and pig this correlation differed to some degree from that of the other animals. In the newborn and adult guinea pigs the correlation was similar. In the rat duodenum the EC density and the 5 HT concentration decreased during development. During growth the correlation remained in principle unchanged although also variations were observed. In the cow the correlation between the EC and 5 HT was quite similar in the mucous submucous layer and in the whole intestinal wall. All these observations strongly support the opinion that the greatest amount of the duodenal 5 HT is located in the EC because the EC and mast cells were the only morphological structures that showed a characteristic yellow fluorescence.

Earlier only Koch and Engelhardt (1959) have estimated the 5 HT content in one EC. According to them one jejunal EC of the pig contained  $2.16 \times 10^{-6} \mu\text{g}$  5 HT. The mean value obtained in this study per one duodenal EC of the pig was about twice as high ( $5.9 \times 10^{-6} \mu\text{g}$ ) but however of the same order of magnitude. In the other domestic animals the 5 HT content per EC was quite similar and somewhat larger than in the guinea pig, mouse and rabbit. Only in the rat the 5 HT content per one EC was distinctly lower ( $1.2 \times 10^{-6} \mu\text{g}$ ). These differences between the various species with respect to the 5 HT content could not be observed in the formaldehyde induced fluorescence or in the argyrophilia but they have obviously some effect on the other staining reactions.



If it is assumed that the fluorescent area of the EC is a symmetrical sphere the concentration of cytoplasmic 5 HT in the EC can be calculated. This concentration in the species studied varied between 2.4 and 9.7 mg/ml. In the electron microscopic pictures the enterochromaffin granules are seen distributed diffusely in the cytoplasm and constitute only a small portion of this cell organ (Christie 1955, Wetzstein *et al* 1962, Toner 1964). It is therefore obvious that the 5 HT concentration in the storage granules is several times as large as the above stated values.

## IV EXPERIMENTAL STUDIES

### EFFECT OF RESERPINE ON THE ENTEROCHROMAFFIN CELLS AND 5-HYDROXYTRYPTAMINE IN THE DUODENUM

#### *Reserpine Experiments*

A total number of 45 adult guinea pigs and 77 rats were studied

Reserpine (Serpasil<sup>®</sup> Ciba AG) was given to the adult animals intraperitoneally and the volume of the injection was adjusted to 4.0 ml with saline. A volume of 0.1 ml of reserpine diluted with saline was given to the newborn rats subcutaneously

*1st Reserpine Experiment* — The guinea pigs received the following doses of reserpine: 0.25 mg/kg (5 guinea pigs), 1.0 mg/kg (5), 4.0 mg/kg (5), 30.0 mg/kg (3). All the animals were killed 24 hours after the injection

*2nd Reserpine Experiment* — 4.0 mg reserpine/kg was given to 15 guinea pigs. Five of them were killed 48, five 96 and five 144 hours after the injection

*3rd Reserpine Experiment* — 2.5 mg reserpine/kg was given to 11 adult rats and 10 mg/kg to 7 adult rats. All the animals were killed 24 hours after the injection

*4th Reserpine Experiment* — An injection of 5.0 mg reserpine/kg (7 adult rats), 15.0 mg/kg (4) or 30.0 mg/kg (10) was given twice 48 and 24 hours before killing

*5th Reserpine Experiment* — 20 mg reserpine/kg was given to 10 rats 1 day of age 24 hours before killing

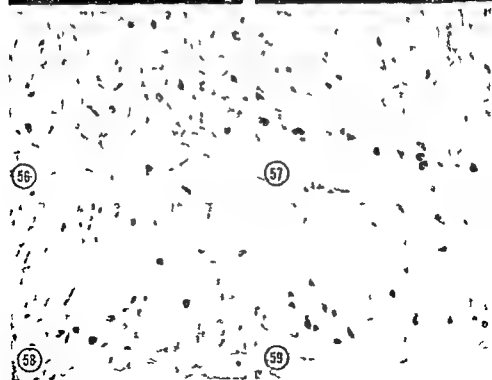
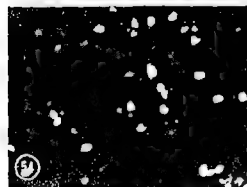
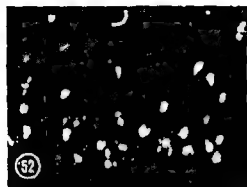
The treatment of the controls was similar to that of the experimental animals with the exception that the injections contained only physiological NaCl. Ten adult guinea pigs and 21 adult and 10 two-day old rats were used as controls

Already with the small doses of reserpine the animals showed a typical sedation, eyelid ptosis and piloerection. With the larger doses a moderate degree of diarrhoea and often haemorrhages in the gastrointestinal tract were observed

The mortality was 50% in the guinea pigs given the dose of 30.0 mg reserpine/kg about 20% in the adult rats given the dose of 30.0 mg/kg twice and about 60% among the newborn rats that received 20.0 mg reserpine/kg

The 5-HT content of the duodenum was determined in all the animals. The number of EC was counted in the duodenum of all the animals in groups 1, 2 and 5 and in their controls. The quantitative estimation of EC was also made for six rats which received the dose of 30.0 mg reserpine/kg

The staining reactions were made in all the animals in groups 1, 2 and 3 as well as in six rats which received the dose of 30.0 mg reserpine/kg. The formaldehyde induced fluorescence reaction was studied in all the experiments

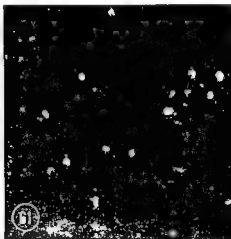
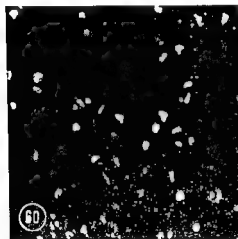


## GUINEA PIG

### *Qualitative Studies*

**Formaldehyde Induced Fluorescence** — Under reserpine action the fluorescent EC exhibited a similar bright yellow colour as the EC of the untreated animals. Also the distribution of the EC was similar to that of the controls and the largest patterns were always seen in the basal parts of the mucosa. The fluorescence in the mast cells did not weaken essentially under the action of reserpine. The greenish fluorescence of the nerve plexus became very weak already after the lowest dose of reserpine.

After the doses of 0.25 and 1.0 mg reserpine/kg the EC exhibited quite similar fluorescence properties as those of the controls (Figs 52—54). Still after the dose of 4.0 mg/kg the main bulk of the EC were strongly fluorescent (Fig 55). However the weakly fluorescent EC were then more numerous than in the controls. After the treatment with 30.0 mg/kg the general fluorescence intensity of the EC was weak (Figs 60 and 61). Two to six days after the reserpine injection of 4.0 mg/kg no essential difference in the fluorescence intensity of the EC was observed between the experiments and controls.



Formaldehyde induced fluorescence in duodenal EC of the guinea pig 24 hours after reserpine treatment  $\times 145$

Fig 60 Control

Fig 61 30.0 mg reserpine/kg. The fluorescence intensity of the EC is obviously decreased

Formaldehyde induced fluorescence and pyrophil reactions in duodenal EC of the guinea pig 24 hours after reserpine treatment  $\times 125$

Fig 52 Control

Fig 53 0.25 mg reserpine/kg. Numerous strongly fluorescent EC are seen

Fig 54 1.0 mg reserpine/kg. Numerous strongly fluorescent EC are seen

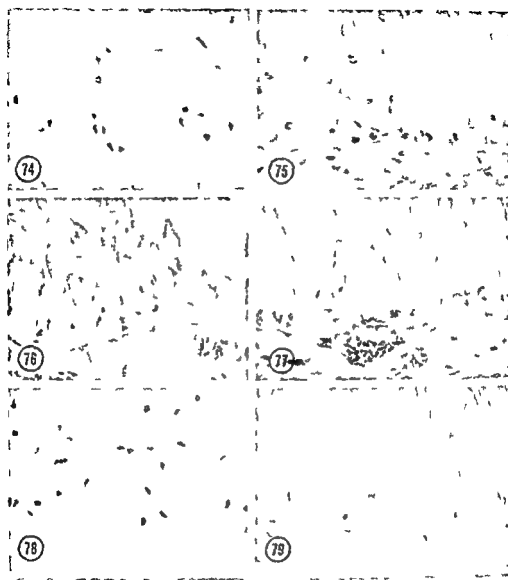
Fig 55 4.0 mg reserpine/kg. Numerous strongly and some weakly fluorescent EC are seen

Fig 56 Control

Fig 57 0.25 mg reserpine/kg. Numerous strongly stained EC are seen

Fig 58 1.0 mg reserpine/kg. Numerous strongly stained EC are seen

Fig 59 4.0 mg reserpine/kg. Numerous strongly stained EC are seen



**Argyrophil Reaction** — After all the doses of reserpine the argyrophil reaction remained intensively positive in the LC (Figs 56—59 62 and 63)

**Other Staining Reactions** — 0.25 mg reserpine/kg In the argentaffin and ferric ferricyanide reactions most of the positive EC were stained as in the controls (Figs 66 67 70 and 71) In the diazo coupling reaction there were numerous weakly stained and some strongly positive EC (Figs 74 and 75) The indophenol reaction showed only some positive EC (Figs 78 and 79)

1.0 mg reserpine/kg Only some strongly stained EC were seen in the argentaffin and ferric ferricyanide reactions (Figs 68 and 72) Most of the EC were not stained specifically In the diazo coupling reaction a few EC stained weakly (Fig 76) The indophenol reaction was very weakly positive only in few EC (Fig 80)

4.0 mg reserpine/kg All the reactions were negative in the EC (Figs 64—65 69 73 77 and 81)

2 days after reserpine treatment (4.0 mg/kg) In the argentaffin and ferric ferricyanide reactions some moderately stained EC were seen but some specimens were negative The diazo coupling reaction showed very few weakly stained EC The indophenol reaction was negative

4 days after reserpine treatment (4.0 mg/kg) The main bulk of the EC were strongly stained in the argentaffin and ferric ferricyanide reactions although several weakly coloured EC were seen In the diazo coupling and especially in the indophenol reaction the number of strongly positive EC was small

6 days after reserpine treatment (4.0 mg/kg) In the argentaffin and ferric ferricyanide reactions the EC were stained as strongly as in the controls In the diazo coupling and especially in the indophenol reaction the number of weakly stained EC was still larger than in the controls

### *Quantitative Studies*

No essential difference in the diameter of the EC was observed in the control and experimental animals Therefore the diameter of  $11.7 \mu$  was used for the Floderus formula in all the experimental groups

The decrease in the number of fluorescent EC was quite linear to the logarithm of the reserpine dose (Fig 82) The dose of 30.0 mg reserpine/kg caused an about 57 % decrease in the EC count

Even the lowest dose of reserpine caused a remarkable decrease (36.5 %) in the 5 HT concentration in the duodenum With increasing doses the decrease

*Diazo coupling and indophenol reactions in duodenal EC of the guinea pig 24 hours after reserpine treatment  $\times 125$*

Fig. 4 Diazo coupling reaction Control

Fig. 75 Diazo coupling reaction 0.25 mg reserpine/kg Some strongly and numerous weakly stained EC are seen

Fig. 6 Diazo coupling reaction 1.0 mg reserpine/kg Only a few specifically stained EC are seen

Fig. 17 Diazo coupling reaction 4.0 mg reserpine/kg No specifically stained EC are seen

Fig. 8 Indophenol reaction Control

Fig. 1 Indophenol reaction 0.25 mg reserpine/kg A few stained EC are seen

Fig. 80 Indophenol reaction 1.0 mg reserpine/kg Only very few EC are just visible

Fig. 91 Indophenol reaction 4.0 mg reserpine/kg No specifically stained EC are seen

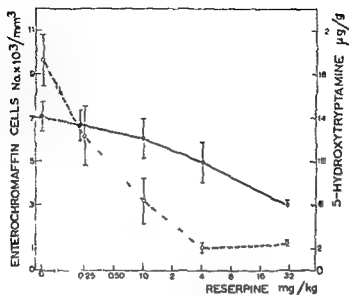


Fig. 82 The logarithmic dose response curve of reserpine on the EC number and the 5-HT concentration in the duodenum of the guinea pig. Ordinate left EC No.  $\times 10^3/\text{mm}^3$  duodenal tissue Ordinate right 5-HT concentration  $\mu\text{g/g}$  duodenal tissue Abscissa reserpine dose mg/kg given 24 hours before killing EC marked —●—●—●— 5-HT marked —○—○—○— Each point is a mean value  $\pm$  standard deviation

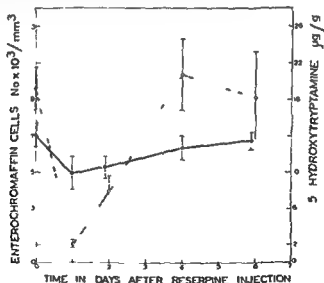


Fig. 83 The effect of reserpine on the EC number and the 5-HT concentration in the duodenum of the guinea pig. Ordinate left EC No.  $\times 10^3/\text{mm}^3$  duodenal tissue Ordinate right 5-HT concentration  $\mu\text{g/g}$  duodenal tissue Abscissa time in days after a reserpine dose of 4.0 mg/kg EC marked —●—●—●— 5-HT marked —○—○—○— Each point is a mean value  $\pm$  standard deviation

was quite linear and the basal mean value ( $2.1 \mu\text{g } 5\text{ HT/g tissue}$ ) was obtained with the dose of  $4.0 \text{ mg reserpine/kg}$ . The dose of  $30.0 \text{ mg/kg}$  was not able to reduce further the  $5\text{ HT}$  concentration as is seen in Fig. 82.

The reappearance of the fluorescent EC after a single reserpine dose of  $4.0 \text{ mg/kg}$  is seen in Fig. 83. The number of cells reached the control level slowly within six days after the injection. On the other hand, the  $5\text{ HT}$  content increased very rapidly (Fig. 83) and the control level was reached within four days.

In all the reserpinized guinea pigs the standard deviations in the EC counts were of the same order (about 12 %) as in the controls. During the reappearance of  $5\text{ HT}$  after reserpine treatment the standard deviations were generally much greater than in the controls.

## RAT

### *Qualitative Studies*

**Formaldehyde Induced Fluorescence** — After each dose of reserpine the fluorescent EC were seen distinctly. No significant weakening in the fluorescence intensity was observed in the adult rat even after a large dose of reserpine ( $30.0 \text{ mg/kg}$  given twice).

**Staining Reactions** — After all the doses of reserpine EC were strongly stained in the argyrophil reaction. In the argentaffin, ferric ferricyanide and diazo coupling reactions there were rather more positive EC than in the controls. Because of the random nature of these reactions in the demonstration of the EC in the rat, no precise counts were made. The indophenol reaction was negative as in the controls.

### *Quantitative Studies*

The diameter of the EC, determined for the Floderus correction, was  $9.7 \mu$  in the adult untreated rat,  $9.8$  in the adult rat given  $30.0 \text{ mg reserpine/kg}$  twice and  $9.8$  in the untreated and the reserpinized newborn rat.

The doses of  $2.5$  and  $10.0 \text{ mg reserpine/kg}$  given once or the doses of  $5.0$  and  $15.0 \text{ mg/kg}$  given twice were not able to reduce significantly the  $5\text{ HT}$  concentration in the duodenum of the rat (Fig. 84). However, the maximum dose of  $30.0 \text{ mg/kg}$  given twice caused a considerable decrease in the  $5\text{ HT}$  content (50.2 %) which was highly significant ( $P < 0.001$ ).

Since the smaller doses of reserpine had no apparent influence on the EC fluorescence and the  $5\text{ HT}$  concentration in the duodenum of the rat, the EC counts were made only after the highest reserpine dose ( $30.0 \text{ mg/kg}$  given twice). After this treatment the decrease in the number of the fluorescent EC was not significant, being about 5 % (Fig. 85).

In the newborn rats a very significant decrease (63.8 %) was observed in the  $5\text{ HT}$  concentration after a large reserpine dose ( $20.0 \text{ mg/kg}$ ). After this treatment the fluorescence intensity of the EC became distinctly weaker than that in the controls. However, the cells were still clearly visible and no essential difference was observed in the number of EC (Fig. 85).



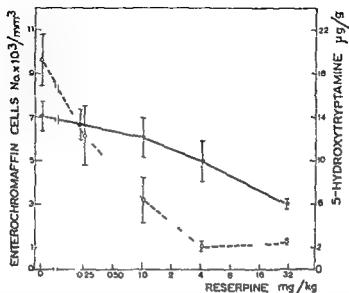


Fig 82 The logarithmic dose response curve of reserpine on the EC number and the 5-HT concentration in the duodenum of the guinea pig. Ordinate left EC No  $\times 10^3/\text{mm}^3$  duodenal tissue. Ordinate right 5-HT concentration  $\mu\text{g/g}$  duodenal tissue. Abscissa reserpine dose mg/kg given 24 hours before killing. EC marked — — — — — 5-HT marked —○—○—○—○— Each point is a mean value  $\pm$  standard deviation.

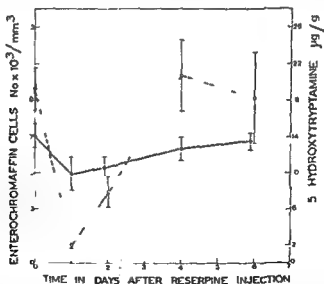


Fig 83 The effect of reserpine on the EC number and the 5-HT concentration in the duodenum of the guinea pig. Ordinate left EC No  $\times 10^3/\text{mm}^3$  duodenal tissue. Ordinate right 5-HT concentration  $\mu\text{g/g}$  duodenal tissue. Abscissa time in days after a reserpine dose of 4.0 mg/kg. EC marked —○—○—○—○— 5-HT marked — — — — — Each point is a mean value  $\pm$  standard deviation.

# EFFECT OF CORTISONE ON THE ENTEROCHROMAFFIN CELLS AND 5 HYDROXYTRYPTAMINE IN THE DUODENUM

## Material

A total number of 24 newborn rats were studied. Cortisone acetate (Adreson N V Organon) was diluted with saline. A daily injection of 0.5 mg cortisone in a volume of 0.1 ml was given subcutaneously on 5–6 days. The first injections were given at the age of 1 day. The controls received 0.1 ml of physiological NaCl daily. The body weight of the experiments (5–8 g) did not clearly differ from that of the newborn rats (5–6 g) and was distinctly smaller than that of the controls (14–17 g).

## Results

The EC diameter determined for the Floderus correction was  $9.8 \mu$  in both the experimental and control animals.

On the fluorescence properties size, shape, granularity and distribution of EC, the chronic cortisone treatment had no essential effect. Few similarly fluorescent mast cells were seen. This kind of yellow fluorescence was not noticed in the other tissue components.

The mean number of fluorescent EC ( $8.670/\text{mm}^2$  tissue) after cortisone treatment was significantly greater ( $P < 0.001$ ) than that of the controls ( $5.790/\text{mm}^2$ ) or that of the newborn rats ( $5.260/\text{mm}^2$ ) as is seen in Fig. 85. Cortisone injections had a similar effect on the 5-HT concentration. The respective mean values were  $13.6 \mu\text{g}$  5-HT/g tissue in the experiments,  $7.9 \mu\text{g}$  in the controls and  $10.8 \mu\text{g}$  in the newborn untreated rats (Fig. 85).

## DISCUSSION

### *Reserpine Studies*

Pletscher *et al.* (1955) found that 5-HT could be liberated from the gastrointestinal tract by reserpine. Benditt and Wong (1957) observed that in the argentaffin and diazo coupling reactions the EC decrease and the 5-HT release were quite linear to the logarithm of the reserpine dose in the duodenum of the guinea pig. They also observed the coincident reappearance of the EC and 5-HT. Later it was demonstrated that all the staining reactions with the exception of the argyrophil reaction became negative in the EC after reserpine treatment (Zbinden *et al.* 1957; Lillie *et al.* 1959; Prellwitz 1959).

In the present study the disappearance and reappearance of the EC and 5-HT was confirmed in the duodenum of the guinea pig after reserpine treatment. It was further noticed that reserpine first abolished the diazo coupling and indophenol reactions in the EC. Already the smallest dose of reserpine turned almost all LC indophenol negative. The diazo coupling and indophenol reactions also reappeared in the EC more slowly than the argentaffin and ferric ferri-cyanide reactions. On the other hand, even after the largest dose of reserpine the argyrophil granules were as strongly positive as in the controls, indicating that the argyrophil reaction must be very sensitive or that it is rendered positive by other properties of the EC than those causing the other staining reactions. These

experimental results as well as the results obtained with untreated animals indicate that the staining of the EC does not reflect the duodenal 5-HT concentration alone and that the diazo coupling and indophenol reactions demonstrate the EC less effectively than the argentaffin and ferric ferri cyanide reactions.

The effect of reserpine on the fluorescence reaction in the EC has not been investigated earlier. The present study showed that even a relatively large dose of reserpine (0.25–4.0 mg/kg) was not able to reduce essentially the fluorescence intensity of the EC. However after the largest dose (30.0 mg/kg) the average fluorescence intensity of the EC became distinctly weaker. The mean concentrations of 5-HT in one EC were  $2.9 \times 10^{-6}$  (control),  $1.9 \times 10^{-6}$  (0.25 mg reserpine/kg),  $1.0 \times 10^{-6}$  (1.0 mg/kg),  $0.4 \times 10^{-6}$  (4.0 mg/kg) and  $0.7 \times 10^{-6}$  (30.0 mg/kg). Two, four and six days after reserpine treatment (4.0 mg/kg) the mean 5-HT contents of the EC were 1.6, 3.5 and  $2.6 \times 10^{-6}$   $\mu$ g. These results suggest that the fluorescence intensity does not reflect the 5-HT concentration of the EC in a linear manner.

The number of fluorescent EC after reserpine treatment was linear to the logarithm of the reserpine dose. After the largest dose however 43% of the EC were still seen. This indicates the high sensitivity of the formaldehyde induced fluorescence in demonstrating EC in comparison to the staining reactions. In contrast to the staining reactions the disappearance and reappearance of the EC and of 5-HT after reserpine treatment occurred strikingly concomitantly in the fluorescence reaction.

The slight effect of large doses of reserpine on the 5-HT concentration in the rat intestine (Lrspamer 1956; West 1958; Karki and Paasonen 1959) was also observed in the present study. Twenty-four hours after the reserpine injections (2.5–15.0 mg/kg) the 5-HT level in the duodenum remained quite similar to that of the controls. Only after the dose of 30.0 mg/kg a distinct decrease of 50.2% was observed in the adult rat. In the newborn rat 5-HT was more easily liberated and a single dose of 20.0 mg/kg already caused a 63.8% release of 5-HT. Obviously the traces of the amines are bound more tightly in the EC granules or the chemical binding mechanism is dissimilar to that of the main bulk of the amines which probably explains the different reserpine effect on 5-HT in the guinea pig and newborn rat in comparison to the adult rat. The synthesis of 5-HT can occur also more rapidly in the rat than in the guinea pig.

In the rat the otherwise weak staining reactions in the EC did not disappear after reserpine treatment (Marks *et al.* 1958; Ghiringhelli and Miri 1959). The present study lends further support to these observations. Even the dose of 30.0 mg/kg did not abolish the staining of EC in the rat duodenum. On the contrary after the smaller doses the stained EC were more numerous than in the controls. Reserpine had no effect on the fluorescence properties of the EC in the adult rat whereas in the newborn rat the fluorescence intensity weakened to some degree after reserpine treatment indicating dissimilar attitudes of the EC in these age groups. However under reserpine action the EC counts remained at both ages quite similar to those of the controls.

It was reported by Zbinden *et al.* (1957) that reserpine had a distinct effect on the staining reactions in the EC and on the 5-HT concentration in the pyloric part of the rabbit stomach but no effect in the fundic part. Also in various organs and species the EC behaved dissimilarly towards reserpine (Lrspamer 1961).

Both these observations as well as the dissimilar attitude of the EC to reserpine in the guinea pig and in the adult and newborn rat, differences in the chemical nature or physiological function of the EC granules in the various gastrointestinal parts, age groups and species.

### *Cortisone Studies*

According to West (1958) small amounts of cortisone given during two weeks had no effect on the duodenal 5-HT content in the adult rat. Later however Telford and West (1960) demonstrated that cortisone and fluorocortisone reduced 5-HT in the small intestine of the rat. The effect of cortisone on the duodenal EC has not been studied earlier.

It was observed in the present study that in comparison to the controls the chronic cortisone treatment caused a significant increase of the duodenal EC density (50 %) and the 5-HT concentration (71 %) in the newborn rat. Also in comparison to the body weight which was of a similar size class in the experiments and untreated newborn rats the duodenal EC density and the 5-HT content increased during cortisone treatment. Possibly cortisone accelerates in some way the functional maturity of EC and causes the increase of the EC density and the 5-HT content (Moog and Richardson 1955). Also the physiological function of the intestine is checked to some degree because of the lethargic condition of the animals and 5-HT is then obviously accumulated in EC. On the fluorescence properties of the EC cortisone had no effect.

The cortisone dose however was so great that its effect on the EC and 5-HT was obviously quite unspecific and to some extent due to the very striking dehydration in the body. Therefore it is difficult to speak of a specific effect of cortisone on the EC and 5-HT. Lempinen (1964) found that cortisone specifically prevented the disappearance of the extra-adrenal chromaffin tissue and even created new chromaffin cells. These observations give reason for the assumption that the extra-adrenal chromaffin tissue and EC have dissimilar functions and are not regulated by the same mechanisms.

In the cortisone experiments the correlation between the EC and 5-HT remained quite similar to that in the controls. This supports Crampers (1958) hypothesis that 5-HT is concentrated in the EC.

## SUMMARY

### 1 *Histochemical Reactions of Enterochromaffin Cells*

In the duodenal EC of the sheep cow pig horse guinea pig mouse rat and rabbit the formaldehyde induced fluorescence and five staining reactions were studied. The fluorescence method was observed to be the most specific and sensitive one. Among the staining reactions the argyrophil method in spite of its poor specificity coloured the EC most effectively.

Most of the EC were found in the mucous layer very few of them in the submucosa and none of them in the other layers of the duodenum.

The formaldehyde induced fluorescence was intense and yellow in the EC of all species. The EC of all the animals exhibited also an intense argyrophilia. When the fluorescence and argyrophilia were consecutively seen in the same sections it was observed that all the fluorescent EC were also argyrophil. The argentaffin reaction stained all the EC in the sheep cow pig and guinea pig. In the duodenum of the other species only several or some of the fluorescent EC were argentaffin.

The staining ability of the EC towards the ferric ferricyanide diazo coupling and indophenol reactions varied considerably. Only in the pig and guinea pig were these three reactions positive in all the fluorescent EC. In the other species these reactions were in general moderate or weak and only some EC were stained. The duodenal EC of the horse mouse and rat were entirely indophenol negative.

The sections examined by both the fluorescence and the staining reactions showed no stained EC that did not have the yellow fluorescence properties.

The EC of none of the species investigated showed autofluorescence in the freeze dried duodenum.

### 2 *Enterochromaffin Cells and 5 Hydroxytryptamine in the Mammalian Duodenum*

The number of fluorescent EC ( $\text{No/mm}^2$  duodenal tissue) and the 5 HT concentration ( $\mu\text{g/g}$  duodenal tissue) were determined from adjacent duodenal tissue pieces taken near the pylorus of the above mentioned eight adult mammalian species as well as from the developing duodenum of the guinea pig and the rat.

Numerous EC were observed in the guinea pig and sheep a few in the horse and moderate amounts in the other animals. The 5 HT concentration was high in the duodenum of the sheep and guinea pig and markedly low in the horse and rat whereas all the other animals showed moderate amounts of 5 HT.

The correlation between the EC number and the 5 HT concentration was highly significant ( $P < 0.001$ ).

In the newborn guinea pig the EC count and the 5 HT concentration were similar to those of the adults. On the other hand in the newborn rat both the EC number and the 5 HT content were significantly above the adult level which was reached at the age of 24—32 days.

The mean 5 HT contents of the EC were greatly variable the standard deviations being 12.3—47.1 %. The highest values were observed in the pig and in the other domestic animals and the lowest value in the rat.

### 3 Experimental Studies

The effect of reserpine on the fluorescence and staining reactions as well as on the EC number and the 5 HT concentration was studied in the duodenum of the guinea pig and rat. The effect of cortisone on the duodenal EC and 5 HT was investigated only in the newborn rat.

**Reserpine** — In the guinea pig the argyrophilia of the EC remained uninfluenced under reserpine action. Reserpine abolished more effectively the diazo coupling and indophenol reactions than the argentaffin and ferric ferricyanide reactions but had a weak effect on the formaldehyde induced fluorescence. The disappearance and reappearance of the staining reactions was linear both to the logarithm of the reserpine dose and to the time after the injections. In the fluorescence reaction the EC number and the 5 HT content decreased and increased concomitantly but on the other hand linearly in the staining reactions.

In the adult rat reserpine caused no essential histochemical alterations in the EC whereas in the newborn rat the formaldehyde induced fluorescence reaction weakened under reserpine action. The largest doses reduced the 5 HT concentration significantly without causing any alterations in the EC number of the adult or the newborn rat.

**Cortisone** — The chronic cortisone treatment had a significantly increasing effect on both the EC number and the 5 HT concentration in the newborn rat. The fluorescence properties of the EC remained uninfluenced.

### 4 Conclusions

The similar size, shape, coarse granularity, yellow fluorescence and strong argyrophilia of all the EC and their identical distribution in the intestinal wall of the mammalian species investigated suggest that the enterochromaffin system is morphologically and functionally similar in the different species and that there is only one principal type of EC in adult mammals.

Towards the other staining reactions the enterochromaffin granules showed a variable affinity and there were also species differences. The sensitivity of the staining reactions in demonstrating the EC decreased in the following order: argyrophil, argentaffin, ferric ferricyanide, diazo coupling and indophenol reaction. In the guinea pig reserpine had a marked influence on these staining reactions, a weak effect on the fluorescence and no effect on the argyrophil properties.

The studies of the EC count and the 5 HT concentration in the duodenum of the adult mammals in the developing duodenum of the guinea pig and the rat in the different layers of the cow duodenum and in the cortisone experiments support the hypothesis that the EC number and the 5 HT concentration are in a direct correlation to each other in the mammalian duodenum. The histochemical and pharmacological studies tend also to show that most of the duodenal 5 HT is located in the EC.

## REFERENCES

- ADAM K. R. and C. WEISS Distribution of 5-hydroxytryptamine in scorpion venoms *Nature (Lond)* 1959 183 1398—1399
- ASTRO B. V. COLO V. ERSPANER and A. VERCELLONE Synthese des Enteramins (5 Oxy tryptamin) *Justus Liebigs Ann Chem* 1952 576 69—74
- BAKER R. V. The intracellular localization of 5 hydroxytryptamine (5 HT) in relation to mitochondrial enzymes *J Physiol (Lond)* 1958a 143 80P
- BAKER R. V. Observations on the localization of 5 hydroxytryptamine *J Physiol (Lond)* 1958b 142 563—570
- BAKER R. V. Mitochondria and storage granules for 5 hydroxytryptamine *J Physiol (Lond)* 1959 145 473—481
- BARKA T. and P. J. ANDERSON *Histochemistry Theory Practice and Bibliography* Harper & Row Publishers Inc New York Evanston London 1963
- BARNETT R. J. P. HAGEN and F. L. LEZ. Mast cell granules containing 5-hydroxytryptamine histamine and heparin morphologically and biochemically distinct from mitochondria *Biochem J* 1958 69 36P
- BARTER R. and A. G. E. PEARSE Detection of 5 hydroxytryptamine in mammalian entero chromaffin cells *Nature (Lond)* 1953 177 810
- BARTER R. and A. G. P. PEARSE Mammalian enterochromaffin cells as the source of serotonin (5-hydroxytryptamine) *J Path Bact* 1955 69 25—31
- BENDITT E. P. and R. L. WONG On the concentration of 5 hydroxytryptamine in mammalian enterochromaffin cells and its release by reserpine *J exp Med* 1957 105 509—520
- BENDITT E. P. R. L. WONG M. ARASH and E. ROEMER 5 Hydroxytryptamine in mast cells *Proc Soc exp Biol (N Y)* 1955 90 303—304
- BERTACCINI G. Osservazioni sul contenuto in 5 idrossitriptamina di vari tessuti del ratto durante lo sviluppo post natale *Ric Sci* 1958 28 2261—2263
- BERTACCINI G. Effect of convulsant treatment on the 5 hydroxytryptamine content of brain and other tissues of the rat *J Neurochem* 1959 4 217—222
- BEATLER A. B. FALCK and Ch. OWMAN Studies on 5 hydroxytryptamine stores in pineal gland of rat *Acta physiol scand* 1964 63 Suppl 239 1—18
- BLASCHKO H. J. M. HAGEN and P. HAGEN Mitochondrial enzymes and chromaffin granules *J Physiol (Lond)* 1957 139 316—322
- BLOCH C. E. Anatomische Untersuchungen uber den Magen Darmkanal des Sauglings *Jb Kinderheilk* 1903 58 121—174
- BODIAN D. A new method for staining nerve fibers and nerve endings in mounted paraffin sections *Anat Rec* 1936 65 89—97
- BODIAN D. The staining of paraffin sections of nervous tissue with activated protargol The role of fixatives *Anat Rec* 1937 69 153—162
- BOGDANSKI D. F. L. BONOMI and B. B. BRODIE Occurrence of serotonin and catecholamines in brain and peripheral organs of various vertebrate classes *Life Sciences* 1963 1 80—84
- BORN G. V. R. and R. E. GILLSON Studies on the uptake of 5-hydroxytryptamine by blood platelets *J Physiol (Lond)* 1959 146 472—491
- BOWMAN R. L. P. A. CAULFIELD and S. UDENFRIEND Spectrophotofluorometric assay in the visible and ultraviolet. *Science* 1955 122 32—33
- BULBRING E. and A. CREMA The release of 5-hydroxytryptamine in relation to pressure exerted on the intestinal mucosa *J Physiol (Lond)* 1959 146 18—28



- BURNER H J and R D LILLIE A five hour variant of Gomori's methenamine silver method for argentaffin cells *Stain Technol* 1949 24 225-227
- CHRISTIE A C A histochemical property of the argentaffin (Kultschitzky) cells *Nature (Lond)* 1954 173 589
- CHRISTIE A C A study of the Kultschitzky (argentaffin) cell with the electron microscope after fixation by osmium tetroxide *Quart J micr Sci* 1955 96 295-299
- CIACCIO C Sur une nouvelle espèce cellulaire dans les glandes de Lieberkühn *C R Soc Biol (Paris)* 1906 60 76-77
- CIACCIO C Sopra speciali cellule granulose della mucosa intestinale *Arch Ital Anat* 1907 6 482-498 Rfr Hamperl 1925
- CLARA M Beiträge zur Kenntnis des Vogeldarmes I Mikroskopische Anatomie *Z mikr anat Forsch* 1926a 4 346-416
- CLARA M Beiträge zur Kenntnis des Vogeldarmes III Die basalgekörnten Zellen im Darm epithel *Z mikr anat Forsch* 1926b 6 28-54
- CLARA M Untersuchungen über die chemische Natur der Körnchen in den basalgekörnten Zellen des Darmepithels bei den Sauropsiden *Z Anat Entwickl Gesch* 1932 98 516-526
- CLARA M Die basalgekörnten Zellen im Darmepithel der Wirbeltiere *Ergeb Anat Entwickl Gesch* 1933a 30 240-340
- CLARA M Über die basalgekörnten Zellen des Darmepithels bei den Amphibien *Z Anat Entwickl Gesch* 1933b 100 76-89
- CLARA M Über die Diazokupplungsreaktion zum Nachweis der *ortho*- und *para*-Phenole in der histologischen Technik *Z histol Mikr* 1934 31 316-337
- CLARA M Untersuchungen über die spezifische Färbung der Körnchen in den basalgekörnten Zellen des Darmepithels durch Benzenfarbstoffe *Z Zellforsch* 1935 22 318-352
- CLARA M and F CANAL Histochemische Untersuchungen an den Körnchen in den basalgekörnten Zellen des Darmepithels *Z Zellforsch* 1932, 15 801-808
- COLF J V J SCHNEIDER and V McHALEN Effect of intraluminal pressure on enterochromaffin cells in the rat duodenum *Proc Soc exp Biol (N Y)* 1961 107 58-59
- COLF J V and A McHALEN Argaffin cells in the intestinal epithelium of human embryo *Nature (Lond)* 1962 193 198
- CORDIER R Recherches morphologiques et expérimentales sur la cellule chromo-argentaffine de l'épithélium intestinal des vertébrés *Arch Biol (Louv)* 1976 36 427-463
- CORDIER R and L ISON Étude histochemique de la substance chromoargentaffine de la cellule de Kultschitzky *Bull Histol Techn micr* 1930 7 140-148
- CORRODI H and N A HILLARY Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 1 Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 6,7-dimethoxyisohinolininderivaten und Formaldehyd *Helv chim Acta* 1963 46 2425-2430
- CORRODI H and N A HILLARY Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 2 Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd *Helv chim Acta* 1964 47 911-918
- CORRODI H N A HILLARY and G JOHNSON Fluorescence methods for the histochemical demonstration of monoamines 3 Sodium borohydride reduction of the fluorescent compounds as a specificity test *J Histochem Cytochem* 1964 12 582-586
- CORRODI H and G JOHNSON Fluorescence methods for the histochemical demonstration of monoamines 4 Histochemical differentiation between dopamine and noradrenaline in models *J Histochem Cytochem* 1965 13 484-487
- COULPLAND R F and J F RILEY Mast cells and 5 hydroxytryptamine in precancerous mouse skin *Nature (Lond)* 1960 187 1128-1129
- COULPLAND R F and I B HEATH Chromaffin cells mast cells and melanin II The chromaffin cells of the liver capsule and gut in ungulates *J Endocrin* 1961 27 71-76
- DAHLSTROM A and K FURER Evidence for the existence of monoamine containing neurons in the central nervous system 1 Demonstration of monoamines in the cell bodies of brain stem neurons *Vita physiol scand* 1964 12 Suppl 232 1-55
- DALGETT C J L C FOLL and T S WONG Fractionation of the smooth muscle stimulants present in extracts of gastrointestinal tract Identification of 5 hydroxytryptamine and its distinction from the substance P *J Physiol (Lond)* 1953 120 298-310
- DANIELI F Zur Histogenese der sogenannten Apudkarzinomide *Ber path Anat* 1974 72 (87-70)

- DAWSON A B A note on the argentaffin cells of the gastric mucosa of the rat *Anat Rec* 1944 89 287—294
- DAWSON A B Argentaffin cells of the gastric mucosa of the rabbit guinea pig mouse and hamster *Anat Rec* 1945 91 53—63
- DAWSON A B Argentophyle and argentaffin cells in the gastric mucosa of the rat *Anat Rec* 1948 100 319—330
- DAWSON A B and I BARNETT Bodian's protargol method applied to other than neurological preparations *Stain Technol* 1944 19 115—118
- DESCIENER E B Argentaffin cell incidence in the rectal mucosa of man mouse and hamster *Nature (Lond)* 1965 207 873—874
- EDER M B MARKLS and K. LOEWER Zur Funktion des enterochromaffinen Zellsystems des Darms im Experiment *Beitr path Anat* 1959 121 50—63
- EDER M H WILLE and O MIDORIKAWA Der Einfluss der Fixation bei histochemischen Untersuchungen an enterochromaffinen Zellen und Panethschen Zellen *Histochemie* 1962 3 54—64
- EELÖF H Chondriosomenstudien an den Epithel und Drüsenzellen des Magen Darmkanals und der Oesophagusdrüsenzellen bei Säugetieren *Anat H* 1914 51 1—228
- ELLENBERGER W *Handbuch der vergleichenden mikroskopischen Anatomie der Haustiere* Bd III Paul Parey Berlin 1911
- FRANKO O On the histochemistry of the rat adrenal medulla *Acta physiol scand* 1951 25 Suppl ■ 22—23
- FRANKO O On the histochemistry of the adrenal medulla of the rat with special reference to acid phosphatase *Acta anat (Basel)* 1952 16 Suppl 17 1—60
- FRANKO O Quenching of tissues for freeze drying *Acta anat (Basel)* 1954 22 331—336
- FRANKO O Distribution of fluorescing islets adrenaline and noradrenaline in the adrenal medulla of the hamster *Acta endocr (Kbb)* 1955a 18 174—179
- FRANKO O *Quantitative Methods in Histology and Microscopic Histochemistry* S Karger Basel New York 1955b
- FRANKO O Personal communication to Dr Falck acknowledged in Falck and Torp (1961)
- FRANKO O Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour *J Histochem Cytochem* 1964 12 487—488
- FRANKO O and L. KAUKO Uptake of monoamines by mesenteric mast cells of the mouse *Acta physiol scand* 1965 64 283—284
- ERIKSEN N G M MARTIN and E P BENDITT Oxidation of the indole nucleus of 5 hydroxy tryptamine and the formation of pigments Isolation and partial characterization of a dimer of 5 hydroxytryptamine *J Biol Chem* 1960 235 1662—1667
- EROS G Über die Bedeutung der argentaffinen Zellen *Frankfurt Z Path* 1930 40 155—160
- EROS G Eine neue Darstellungsmethode der sogenannten 'gelben' argentaffinen Zellen des Magendarmtraktes *Zbl allg Path path Anat* 1932 54 385—391
- ERSPAMER V Cellule enterocromaffini e cellule argentofile nel pancreas dell'uomo e del mammifero *Z Anat Entwickl Gesch* 1937 107 574—619
- ERSPAMER V Caratteristiche delle cellule enterocromaffini e delle cellule preenterocromaffini argentofile *Anat Anz* 1938 86 379—388
- ERSPAMER V Farmacologiche Studien über Enteramin I Über die Wirkung von Aceton extrakten der Kaninchenmagenschleimhaut auf den Blutdruck und auf isolierte überlebende Organe *Naunyn-Schmiedeberg's Arch exp Path Pharmac* 1940a 196 343—365
- ERSPAMER V Farmacologiche Studien über Enteramin III Über das Vorhandensein eines enteraminähnlichen Stoffes in Milzextrakten *Naunyn-Schmiedeberg's Arch exp Path Pharmac* 1940b 196 391—407
- ERSPAMER V Ricerche chimiche e farmacologiche sugli estratti di ghiandola ipobranchiale di *Murex trunculus* *Murex brandaris* e *Tritonalia emacea* IV Presenza negli estratti di enteramina o di una sostanza enteraminosimile *Arch int Pharmacodyn* 1948 76 308—326
- ERSPAMER V Über den 5 Hydroxytryptamin (Enteramin) Gehalt des Magen Darmtraktes bei den Wirbeltieren *Naturwissenschaften* 1953 40 318—319
- ERSPAMER V Quantitative estimation of 5 hydroxytryptamine in gastrointestinal tract spleen and blood of vertebrates *In Ciba Found Symp on Hypertension* Ed G E W Wolstenholme and M P Cameron J & A Churchill Ltd London 1954 78—84
- ERSPAMER V Observations on the 5 hydroxytryptamine (enteramine) release caused by reserpine in the rat *Experientia (Basel)* 1956 12 63—64

- ERSPAMER V Occurrence and distribution of 5 hydroxytryptamine (enteramine) in the living organism *Z Vitamin Hormon u Fermentforsch* 1958 9 74-96
- ERSPAMER V Recent research in the field of 5-hydroxytryptamine and related indolealkyl amines *Fortschr Arzneimittelforsch* 1961 3 151-367
- ERSPAMER V and B ASTRO Isolation of enteramine from extracts of posterior salivary glands of *Octopus vulgaris* and of *Discoglossus pictus* skin *J Biol Chem* 1952a 200 311-318
- ERSPAMER V and B ASERO Identification of enteramine the specific hormone of the enterochromaffin cell system as 5-hydroxytryptamine *Nature (Lond)* 1952b 169 800-801
- ERSPAMER V and G BORETTI Identification of enteramine and enteramine related substances in extracts of posterior salivary glands of *Octopus vulgaris* by paper chromatography *E perientia (Basel)* 1950 6 348-349
- ERSPAMER V and A TESTINI Observations on the release and turnover rate of 5-hydroxytryptamine in the gastrointestinal tract *J Pharm Pharmacol* 1959 11 618-623
- ERSPAMER V and M VIALLI Presence of enteramine in the skin of amphibians *Nature (Lond)* 1951 167 1033
- FALCK B Observations on the possibilities of the cellular localization of monoamines by a fluorescence method *Acta physiol scand* 1962 56 Suppl. 197 1-25
- FALCK B, A BERTLER N, A HILLARP G, ROSENCRON and A TORP Dopamine and chromaffin cells *Acta physiol scand* 1959 47 251-258
- FALCK B, N A HILLARP G, THUREN and A TORP Fluorescence of catecholamines and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962 10 348-354
- FALCK B and CH OWMAN A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines *Acta Univ Lund II* 1965 7 1-23
- FALCK B and A TORP A fluorescence method for histochemical demonstration of noradrenalin in the adrenal medulla. *Med exp (Basel)* 1961 5 429-432
- FAUSTINI R The enteric distribution of 5 hydroxytryptamine (enteramine serotonin) in some large domestic mammals and the appearance of 5 hydroxytryptamine and the enterochromaffin cell system in the embryonic calf *Immunol J vet Res* 1955 16 397-400
- FELDBERG W and C. C. TON Distribution of 5 hydroxytryptamine (serotonin enteramine) in the wall of the digestive tract *J Physiol (Lond)* 1953 119 352-362
- FETTER F Carcinoid und Carcinom. *Ergeb allg Path path Anat* 1934 29 305-499
- FETTER F *Über die peripheren endokrinen (parakrinen) Drüsen des Menschen* Wilhelm Maudrich Wien Dusseldorf 1953
- FILIPPI P DE Le cellule enterocromaffini nei pesci *Boll Soc med chir Paris* 1930 44 441-444
- FRIDMANN I Ein Beitrag zur Kenntnis der basalgelben Zellen des Darmtraktes beim Menschen. *Z mikr-anat Forsch* 1934 36 99-136
- FUNK H U, L. WEBER and CH. HEDINGER Vermehrung der argentaffinen Zellen und Carcinome des Magen Darmtraktes bei Nierenveränderungen *Virchows Arch path Anat* 1966a 340 312-319
- FUNK H U, L. WEBER CH. HEDINGER and TH. HARDMEIER Die Zahl argentaffiner Zellen des menschlichen Verdauungstraktes unter normalen und pathologischen Bedingungen *Virchows Arch path Anat* 1966b 340 289-303
- GEISY A G *Documenta Geigy Wissenschaftliche Tabellen* Ed. K. Dietz Basel 6th ed 1960
- GÉPARD L, R. CORDIER and L. LISON Sur la nature de la fraction chromaffine *C R Soc Biol (Paris)* 1930 105 876-877
- GEFFRON M D, A. B. DRAKONTIDES and L. L. ROSS Serotonin Synthesis and release from the myenteric plexus of the mouse intestine *Science* 1965 149 197-199
- CERZELI G Presence of enterochromaffin cells in the gut of amphioxus *Nature (Lond)* 1961 189 237-238
- GIHRINGHELLI F Sulla presenza di cellule enterocromaffini nella prostata e nella uretra prostatica del cane *Riv Istoclim norm pat* 1956 2 235-239
- GIHRINGHELLI F and I. MIRA Azione della reserpina sulle cellule enterocromaffini del ratto e sulla valutazione qualitativa e quantitativa *Arch Biol (Louv)* 1959 10 875-900
- GIRRI H III Phenol tests III The indophenol test *J Biol Chem* 1927 77 649-664
- CLICK D *Techniques of Histo- and Cytochemistry* Interscience Publishers Inc New York London 1949

- GOLODETZ L and P G UNNA Zur Chemie der Haut III Das Produktionsvermögen der histologischen Element der Haut *Ab prakt Derm* 1909 48 149—166
- GOMORI G Staining of chromaffin tissue *Amer J clin Path* 1941 7 115—117
- GOMORI G Chemical character of the enterochromaffin cells *Arch Path* 1948 45 48—55
- GOMORI G Histochemistry of the enterochromaffin substance *J Histochem Cytochem* 1954 2 50—53
- HALLIER S Über die Wirkung wilder kleiner Gaben von Insulin Eltyran und Tonerphin auf die basalgelagerten Zellen im Darm der weissen Ratte und des Meerschweinchens *Z Zellforsch* 1937 27 52—64
- HAMMARSTROM L M RITZEN and S ULLBERG Combined autoradiography and fluorescence microscopy Localization of labelled 5 hydroxytryptophan in relation to endogenous 5 hydroxytryptamine in the gastrointestinal tract *Experientia* 1966 22 213—215
- HAMON M Cytochemical research on coelenterate nematocysts *Nature (Lond)* 1955 176 357
- HAMPERL H Über die »gelben (chromaffinen)« Zellen im Epithel des Verdauungstraktes *Z mikr anat Forsch* 1925 2 506—535
- HAMPERL H Über die »gelben (chromaffinen)« Zellen im gesunden und kranken Magendarmtrakt *Virchows Arch path Anat* 1927 266 509—548
- HAMPERL H Was sind argentaffine Zellen? *Virchows Arch path Anat* 1932 286 811—833
- HAMPERL H Über argyrophile Zellen. *Virchows Arch path Anat* 1952 321 482—507
- HARDISTY R M G I C INGRAM and R S STACEY Reserpine and human platelet 5 hydroxytryptamine *Experientia (Basel)* 1956 12 424—425
- HARDMEIER TH and CHR HEDINGER Das Verhalten argentaffiner Zellen im Bereich steiniger Dickdarmtumoren *Virchows Arch path Anat* 1965 338 332—339
- HASEGAWA T Über die Carcinome des Wurmfortsatzes und des Dickdarmes *Virchows Arch path Anat* 1923 244 8—37
- HEIDENHAIN R Untersuchungen über den Bau der Labdrüsen *Arch mikr Anat* 1870 6 368—406
- HELLANDER H F A preliminary note on the ultrastructure of the argyrophile cells of the mouse gastric mucosa *J Ultrastruct Res* 1961 5 257—262
- HELLWEG G Über Vorkommen und gegenseitiges Verhalten der argentaffinen und argyrophilen Zellen im menschlichen Magen Darmtrakt *Z Zellforsch* 1952 36 546—551
- HOLCENBERG J and E P BENDITT A new histochemical technique for demonstration of enterochromaffin cells A reaction for indole-ethylamines *J Histochem Cytochem* 1959 7 303—304
- HOLCENBERG J and E P BENDITT A new color reaction for tryptamine derivatives Histochemical application to enterochromaffin cells *Lab Invest* 1961 10 144—158
- HOESCHEN T Über Menge und Verteilung der basalgelagerten Zellen im Darm des Meerschweinchens *Z Zellforsch* 1937 27 711—719
- HUGHES F B R KUNTZMAN and P A SHORE Nature of serotonin binding in tissues *Fed Proc* 1957 16 308
- JACOBSON W The argentaffine cells and pernicious anaemia *J Path Bact* 1939 49 1—19
- JACOBSON W The yellow pigment of the argentaffine cells of the mammalian gastrointestinal tract In *Ciba Found Symp on Chemistry and Biology of Pteridines* Ed G E W Wolstenholme and M P Cameron J & A Churchill, Ltd London 1954 314—328
- JACOBSON W and M M SIMPSON The fluorescence spectra of pterins and their possible use in the elucidation of the antipernicious anaemia factor *Biochem J* 1946 40 3—18
- JEPSON J B and J J STEVENS A fluorescence test for serotonin and other tryptamines *Nature (Lond)* 1953 172 772
- KARAI N T and M K PAASONEN Selective depletion of noradrenaline and 5-hydroxytryptamine from the rat brain and intestine by rauwolfia alkaloids *J Neurochem* 1959 3 352—357
- KARKI N T M K PAASONEN and P PELTOLA Factors influencing the release of 5 hydroxytryptamine from the rabbit intestine into the venous blood *Ann Med exp Fenn* 1960 38 231—236
- KALFMAN WOLF M Kurze Notiz über Belegzellen Panethsche Zellen und basalgelagerte Zellen im Darm des Menschen *Anst An* 1911 39 670—672
- KELLER R Histamin und 5-Hydroxytryptamin in den Gewebemastzellen der Albinoratte *Helv physiol pharmacol Acta* 1957 15 371—375
- KLEMM E Über die Wirkung von Insulin Eltyran und Tonerphin auf die basalgelagerten Zellen im Darm der weissen Ratte *Z Zellforsch* 1937 26 387—395

- KOCH G and G ENGELHARDT Über das argyrophile Hells Zellen Organ in der Darmrohrendschleimhaut des Säugetiers *Naunyn-Schmiedeberg's Arch exp Patb Pharmac* 1959 236 463-473
- KULL H Die chromaffinen Zellen des Verdauungstraktes *J mikr-anat Forsch* 1925 2 163-200
- KULTSCHITZKY N Zur Frage über den Bau des Darmkanals *Arch mikr Anat* 1897 49 7-35
- LADENFLOTT D M PHILLIPS and E P BRIDGES The histochemical demonstration of histamine in mast cells *J Histochem Cytochem* 1961 9 534-541
- LASKER A and J GRIZO Argentaffin cells of the human appendix A comparative study of the results obtained with modified Schmorl and Masson techniques *Arch Patb* 1948 46 83-84
- LEMBECK F 5-Hydroxytryptamine in a carcinoid tumour *Nature (Lond)* 1953 177 910-911
- LEMBECK F and H FELD Serotoninfreisetzung aus isolierten Granula enterochromaffiner Zellen *Naunyn-Schmiedeberg's Arch exp Patb Pharmac* 1961 253 409-420
- LEMPINEN M Extra adrenal chromaffin tissue of the rat and the effect of cortical hormones on it *Acta physiol scand* 1964 62 Suppl 231 1-91
- LILLIE R D *Histopathologic Technique and Practical Histochemistry* The Blakiston Company Inc New York Toronto 1954a
- LILLIE R D Argentaffin and Schiff reactions after periodic acid oxidation and aldehyde blocking reactions *J Histochem Cytochem* 1954b 2 127-136
- LILLIE R D Investigations on the structure of the enterochromaffin substance *J Histochem Cytochem* 1961 9 184-189
- LILLIE R D and H J BURTON The ferric ferrocyanide reduction test in histochemistry *J Histochem Cytochem* 1953 1 87-92
- LILLIE R D H J BURTON and J E GRECO Histochemical reactions in histochemistry *J Histochem Cytochem* 1953 1 154-159
- LILLIE R D and C G GLENNER Histochemical reactions in carcinoid tumors of the human gastrointestinal tract *Amer J Patb* 1960 36 623-651
- LILLIE R D J P GRECO-HINSON and J L CASON Azo-coupling rate of enterochromaffin with various diazonium salts *J Histochem Cytochem* 1961 9 11-21
- LILLIE R D H WEISSBACH and G G GLENNER On the experimental discharge of the gastric and intestinal argentaffin cells *J Histochem Cytochem* 1959 7 23-26
- LISON L Recherches histochimiques sur les phénols et leurs dérivés *Arch Biol (Lige)* 1931 41 343-436
- MARSH B H R W SORREN and I R HAYES Effects of reserpine and chlorpromazine upon enterochromaffin cells of the rat *Proc Soc exp Biol (N Y)* 1958 97 413-415
- MASSON P La glande de l'intestin chez l'homme *C R Acad Sci (Paris)* 1914 158 59-61
- MASSON P Carcinoids (argentaffin-cell tumors) and nerve hyperplasia of the appendicular mucosa *Amer J Patb* 1928 4 181-212
- MOELLER W Anatomische Beiträge zur Frage von der Sekretion und Resorption in der Darmschleimhaut *Zool* 1899 66 69-135
- MONESI V The appearance of enterochromaffin cells in the intestine of the chick embryo *Acta Anat (Basel)* 1960a 41 97-114
- MONESI V Differentiation of argyrophil and argentaffin cells in organotypic cultures of embryo chick intestine *J Embryol exp Morph* 1960b 8 302-313
- MONG F and M RICHARDSON The functional differentiation of the small intestine IV The influence of adrenocortical hormones on differentiation and phosphatase synthesis in the duodenum of the chick embryo *J exp Zool* 1955 130 29-55
- MÜNGH H Über Menge und Verteilung der basophilen Zellen im Darm der weissen Ratte *Z mikr anat Forsch* 1939 45 244-254
- MURRAY J G and J H WYLLIE Distribution of histamine 5-hydroxytryptamine and other pharmacologically active substances in the human stomach *Gut* 1964 5 530-536
- MUTHMANN I Beiträge zur vergleichenden Anatomie der Blinddarm und des lymphoiden Organe des Darmkanals bei Säugetieren und Vögeln *Anat H* 1913 48 65-114
- NICOLA A Recherches sur l'épithélium de l'intestin grêle *Int Microsc Anat Plast (Leipz)* 1891 8 1-58
- NORBERG K A Adrenergic innervation of the intestinal wall studied by fluorescence microscopy *Int J Neuropharmacol* 1964 3 379-382
- PARRAT J R and C B WELSH 5-Hydroxytryptamine and tissue mast cells *J Physiol (Lond)* 1957 137 169-179

- PATZELT V. D. r. Darm. In *Handbuch der mikroskopischen Anatomie des Menschen* Vol. V 3. Ed. W. v. Mollendorff. Julius Springer Berlin 1936 137—152
- PEARSE A. G. E. *Histochemistry Theoretical and Applied* J. & A. Churchill Ltd. London 1953
- PEARSE A. G. E. A contribution to the chemistry of mammalian enterochromaffin cells. *Riv. Istochim. norm. pat.* 1956 2 103—110
- PEARSE A. G. E. *Histochemistry Theoretical and Applied* J. & A. Churchill Ltd. London 1960
- PLETSCHER A. P. A. SHORE and B. H. BRODIE Serotonin release — a possible mechanism of reserpine action. *Science* 1955 122 374—375
- PRELLWITZ W. Der Einfluss des Reserpins und Tryptophans auf das enterochromaffine Gewebe beim Meerschweinchen. *Z. ges. exp. Med.* 1959 131 301—311
- PRETL K. Zur Frage der Endokrinie der menschlichen Vorsteherdrüse. *Virchows Arch. path. Anat.* 1944 312 392—404
- PALSOFF W. H. The distribution of 5-hydroxytryptamine and adenosinetriphosphate in cytoplasmic particles of the dog's small intestine. *Brit. J. Pharmacol.* 1960 15 520—524
- QUARONI E. Presenza di cellule preenterochromaffini argentofili nel timo di pollo. *Riv. Istochim. norm. pat.* 1956 2 185—187
- RATZENHOFER M. and D. LEB. Über die Feinstruktur der argentaffinen und der anderen Erscheinungsformen der »Hellen Zellen« Fevters im Kaninchen-Magen. *Z. Zellforsch.* 1965 67 113—130
- RESNICK R. H. and S. J. GRAY Distribution of serotonin in the human gastrointestinal tract. *Gastroenterology* 1961 41 119—131
- RITZÉN M. L. HANMARSTROM and S. ULLBERG Autoradiographic distribution of 5-hydroxytryptamine and 5-hydroxytryptophan in the mouse. *Biochem. Pharmacol.* 1964 14 313—321
- ROGOSINA M. Beiträge zur Kenntnis des Verdauungskanal der Fische I. Über den Bau des Epithels im Pylorusabschnitt des Magens von *Acipenser ruthenus* L. *Z. mikr.-anat. Forsch.* 1928 14 333—372
- ROGOSINA M. Beiträge zur Kenntnis des Verdauungskanal der Fische II. Über den Bau des Epithels im Kardiaschnitt des Magens von *Acipenser ruthenus* L. *Z. mikr.-anat. Forsch.* 1930 20 298—326
- ROWEIS B. *Mikroskopische Technik* R. Oldenbourg München 1948
- SCHACK L. Über die Gelben Zellen im menschlichen Wurmfortsatz. *Beitr. path. Anat.* 1932 90 441—478
- SCHMIDT J. E. Beiträge zur normalen und pathologischen Histologie einiger Zellarten der Schleimhaut des menschlichen Darmkanals. *Arch. mikr. Anat.* 1905 66 12—40
- SCHUMACHER A. and H. SCHULZ Licht- und elektronenmikroskopische Untersuchungen an einem metastasierenden Dunndarmcarcinom mit Serotoninbestimmungen an Tumorzellfraktionen. *Klin. Wschr.* 1963 41 1188—1196
- SCHUMANN G. Untersuchungen über das Verhalten der basal gelegenen Zellen im Darm des Meerschweinchen in Abhängigkeit vom Lebensalter, Geschlecht und Trächtigkeit. *Z. mikr.-anat. Forsch.* 1939 45 233—243
- SHARPLES W. The histogenesis of the argentaffin cells in the stomach and duodenum of the rat. *Anat. Rec.* 1945a 91 107—117
- SHARPLES W. A note on the relatively high number of argentaffin cells in the mucosa of the human stomach. *Anat. Rec.* 1945b 91 237—243
- SHEPHERD D. M. G. B. WEST and V. ERSKINER Detection of 5-hydroxytryptamine by paper chromatography. *Nature (Lond.)* 1953 172 357
- SINGH I. On the alleged presence of non-argyrophil argentaffin cells in the human gastrointestinal tract. *Z. Zellforsch.* 1963 59 615—624
- SINGH I. Further observations on the alleged presence of non-argyrophil argentaffin cells in the human gastro-intestinal tract. *Z. Zellforsch.* 1964a 67 121—124
- SINGH I. A new argyrophil method for the rapid staining of enterochromaffin cells in paraffin sections. *Acta Anat. (Basel)* 1964b 59 290—296
- SINGH, I. The relative proportion of pre-enterochromaffin (argyrophil) and enterochromaffin (argentaffin) cells in the gastro-intestinal tract of human fetuses. *Z. Zellforsch.* 1965a 67 338—342
- SINGH I. Methods of quantitative estimation of enterochromaffin cells. *Anat. Anz.* 1965b 11 322—327
- THUCLEER E. A. N. ERIKSEN and E. P. BENDITT The chemical nature of the chromation reaction in the enterochromaffin cells. *J. Histochem. Cytochem.* 1960 8 314—315

- SPRATKE H. Untersuchungen über die argentaffinen Zellen in der Schleimhaut des Wurmfortsatzes und ihre Beziehungen zur Entstehung der sogenannten Karzinoide. *Frankf. Z. Path.* 1927 35: 302—319.
- SULLIVAN T. J. The effect of diet on the 5-hydroxytryptamine content of the small intestine and other organs in rats and mice. *Brit. J. Pharmacol.* 1960 15: 513—519.
- TANC L. H. Über die Panethschen Zellen sowie die gelben Zellen des Duodenums beim Schwein und den anderen Wirbeltieren. *Arch. mikr. Anat.* 1922 9: 182—209.
- TEJETER J. Über die enterochromaffinen Zellen der Haussaugetiere. *Z. mikr. anat. Forsch.* 1930 21: 462—491.
- TELFORD J. M. and C. B. WERT. The effects of corticosteroids and related compounds on the histamine and 5-hydroxytryptamine content of rat tissues. *Brit. J. Pharmacol.* 1960 15: 532—539.
- TISSARI A. 5-Hydroxytryptamine 5-hydroxytryptophan decarboxylase and monoamine oxidase during foetal and postnatal development in the guinea pig. *Acta physiol. scand.* 1966 67 Suppl. 265: 1—80.
- TOFFI C. C. Effects of temperature on the 5-hydroxytryptamine (serotonin) content of tissues. *J. Physiol. (Lond.)* 1960 151: 410—415.
- TOFFI C. Die Entwicklung und Ausbildung der Drüsen des Magens. *S. B. Akad. Wiss. Wien math. nat. Kl.* 1880 82: 57—128.
- TONER I. C. Fine structure of argentophil and argentaffin cells in the gastro-intestinal tract of the fish. *J. Morph.* 1964 63: 830—839.
- TURB L. Über enterochromaffine Zellen. *Anat. Anz.* 1929 67: 49—58.
- TÖRO L. Zur Frage der Darmresorption auf Grund von Untersuchungen am Herkoldildarm. *Z. mikr. anat. Forsch.* 1930 19: 537—556.
- UDENFRIEND S. H. WEISSBACH and C. F. CLARR. The estimation of 5-hydroxytryptamine (serotonin) in biological tissues. *J. Biol. Chem.* 1955 215: 337—344.
- VERITY M. A. S. M. MYELINKOFF M. FRANKLAND and M. CRIPPEL. Serotonin content and argentaffin and Paneth cell changes in ulcerative colitis. *Gastroenterol.* 1962 43: 24—31.
- VERNE, J. La réaction chromaffine en histologie et sa signification. *Bull. Soc. Chim. biol. (Paris)* 1923 5: 227—235.
- VETTER J. Experimentelle Untersuchungen über das Verhalten der basalepithelialen Zellen im Darmepithel der dimastizierten Ratte bei verminderter und gesteigerter Vitaminzufuhr. *Z. mikr. anat. Forsch.* 1937 42: 555—577.
- VETTER J. Über das Verhalten der basalepithelialen Zellen bei hungernden Tieren (Meerschweinchen, weiße Ratten und weiße Mäuse). *Z. mikr. anat. Forsch.* 1938 43: 123—132.
- VIALI M. Ricerche sull'intestino del rettili. 4. L'epithelio intestinale. *Arch. Biol. (Lige)* 1929 39: 527—581.
- VIALI M. Contributo alla conoscenza delle ghiandole cutanee e dello strato corneo di *Rana kishinouyei*. *Ann. Ist. Lomb. Sci. Cl. Sci.* 1955 83: 243—249.
- VIALI M. and C. CASATI. Sulla presenza di cellule enterochromaffini nel timo dei rettili. *Atti Ist. chim. norm. pat.* 1958 4: 343.
- VIALI M. and V. CRISPAMER. Cellule enterochromaffini e cellule basiparuloase acidofile nei vertebrati. *Z. Zellforsch.* 1933 19: 743—773.
- VIALI M. and V. CRISPAMER. Considerazioni sul sistema enterochromaffine (A proposito di una recente monografia di Feyrer). *Z. Zellforsch.* 1939 29: 487—501.
- VIALI M. and V. CRISPAMER. Ricerche di caratterizzazione chimica delle sostanze fenoliche presenti negli estratti acetonici di ghiandole salivari posteriori di *Octopus vulgaris*. *Arch. Fisiol.* 1940 40: 293—312.
- WEISSBACH H. The estimation of serotonin in biological material. *Stand. Meth. Clin. Chem.* 1961 4: 197—203.
- WEISSBACH H. T. P. WAHLENS and H. UDENFRIEND. A simplified method for measuring serotonin in tissues: simultaneous assay of both serotonin and histamine. *J. Biol. Chem.* 1958 230: 865—871.
- WEST G. B. Studies on 5-hydroxytryptamine and 5-hydroxytryptophan. *J. Pharm. Pharmacol.* 1958 10: 92—97T.
- WITZSTYEN R. W. DORFIER and A. SCHWAB. Die Feinstruktur der enterochromaffinen Zellen und ihrer spezifischen Granula. Elektronenmikroskopische Untersuchungen am Duodenum des Meerschweinchens. *Protoplasma (Wien)* 1962 55: 303—312.

- ZBINDEN G. V. PLETSCHER and A. STÜDER Regionale Unterschiede der Reserpinwirkung auf enterochromaffine Zellen und 5-Hydroxytryptamin Gehalt im Magendarmtrakt. *Schweiz med Wochschr* 1957 22 629—631
- ZBINDEN G. A. PLETSCHER and A. STÜDER Alimentäre Beeinflussung der enterochromaffinen Zellen und des 5 Hydroxytryptamin Gehalts von Gehirn und Darm. *Z ges e p Med* 1958 129 615—620
- ZIMMERMANN K. W. Beiträge zur Kenntnis einiger Drüsen und Epithelien. *Arch mikr Anat* 1898 52 552—706









**ACTA PHYSIOLOGICA SCANDINAVICA**

**VOL 69 SUPPLEMENTUM 282**

**PIGMENT MIGRATION, LIGHT SCREENING  
AND RECEPTOR SENSITIVITY  
IN THE COMPOUND EYE  
OF NOCTURNAL LEPIDOPTERA**

**BY**

**GUNNAR HÖGLUND**

**STOCKHOLM 1966**



ACTA PHYSIOLOGICA SCANDINAVICA

*Vol 69 Supplementum 282*

FROM THE DEPARTMENT OF PHYSIOLOGY  
KAROLINSKA INSTITUTET  
STOCKHOLM 60 SWEDEN

PIGMENT MIGRATION, LIGHT SCREENING  
AND RECEPTOR SENSITIVITY  
IN THE COMPOUND EYE  
OF NOCTURNAL LEPIDOPTERA

BY

GUNNAR HÖGLUND

STOCKHOLM 1966



# CONTENTS

Introduction	7
Material and Methods	7
Material	7
Dissection	7
Light stimulation	7
Recording of potential changes	9
Determination of threshold response	11
Recording of glow	13
Histological procedures	13
Measurements on fresh eyes	14
Symbols used in the illustrations	14
I Pigment migration and glow	16
Results	16
Discussion	20
II Pigment migration and threshold changes in dark adapted eyes kept in darkness	22
Results	23
Discussion	28
III Pigment migration and threshold changes during and after illumination of the eye	31
Results	31
Pigment remaining in extreme distal position or pigment removed	33
Pigment migrating during the illumination	33
Pigment migrating during the dark interval between the illumination periods	40
Variations in transpigment threshold during pigment migration in the illuminated eye	42
Neutral density filters substituting for pigment screening effect	43
Discussion	45
Summary	50
References	53





## INTRODUCTION

The amount of light reaching the light sensitive parts of the photoreceptor cells may be regulated by several mechanisms in the eye. These mechanisms include movements of the iris and its appendages, the exposure or covering of light reflecting layers, and movements of the receptors or of retinal "screening" pigments.

Pigment movements occur together with other regulatory mechanisms in many animal groups such as birds, reptiles, amphibians and molluscs. In some groups, e.g. in mammals, pigment movements are usually considered not to occur while in the eyes of other forms such as the compound eye of some arthropods, pigment migration is regarded as the only mechanism regulating the intensity of light reaching the receptors. (See Parker 1932 and Duke Elder 1958.)

It has recently been shown that the retinal sensitivity varies with the pigment position in arthropod, mollusc and vertebrate eyes. In the compound eye of night moths the sensitivity increase during dark adaptation often proceeds in two phases. The second of these phases occurs in parallel with the outward pigment movement (Bernhard and Ottoson 1960 a, 1960 b, 1964; Bernhard, Hoglund and Ottoson 1963; Post and Goldsmith 1965). Experimental results indicate that movements of the proximal pigment influence the sensitivity of the compound eye in some crustaceans (de Bruin and Crisp 1957). In the molluscs *Octopus* and *Ommastrephes* the sensitivity increase during dark adaptation has been related to pigment migration (Byzov, Orlov and Utina 1962). Dodd and Jessen (1961) have reported that in the frog the pigment migration occurring when the eye is exposed to an adapting light causes a threshold decrease of more than one log unit. Back, Donner and Reuter (1964) found that the amount of rhodopsin bleached by exposing the frog eye to a standard light varies with the pigment position. The bleaching efficiency increases when the pigment retracts from the rod outer segments. Back, Donner and Reuter concluded that the migration of the pigment from the dark adapted to the light adapted position reduces the effective intensity of the light to  $1/3$ .

The investigations mentioned above provide evidence that the photoreceptor sensitivity is influenced by the pigment position. The experiments described in the present paper were undertaken in an attempt to study the relation between the receptor light sensitivity and the variations in screening effect dur-

ing pigment movements. The experiments were done on the compound eye of night moths (mainly *Geleno euphorbiae* and *Deilephila elpenor*). These species were chosen because both in the light adapted and in the dark adapted state the migrating accessory pigment is located between the corner and the reticular cell rhombom which is presumed to be the primary site of photoreception (see Goldsmith 1964). The anatomical separation between the migrating pigment and the rhomboms was taken advantage of to develop an experimental technique which permitted the receptor sensitivity to be determined not only by light transmitted through the corner and the pigment but also by exposing the receptors directly to a test light. The experiments reported in part I were done to find out if variations in the glow (page 16) can be used as an index of accessory pigment movements. The position of the pigment near the basement membrane in light adapted and dark adapted eyes was also determined. This pigment is believed to be located in the basal pigment cells (Stefanowski 1890, Yagi and Koyama 1963). Part II describes studies of receptor threshold and screening effect during pigment movements in dark adapted eyes. In the experiments described in part III dark adapted eyes were exposed to adapting illuminations of varying intensities and durations. In some experiments the pigment remained in a distal position during the illumination while in other experiments the pigment migrated in the proximal direction. The relation between the receptor threshold and the pigment position under these experimental conditions was studied.

The principal results have been described in a short previous report (Höglund 1963 b). A communication has also been presented at the Symposium on the Functional Organization of the Compound Eye held in Stockholm in 1965 (Höglund 1966).

## MATERIAL AND METHODS

**Material** The experiments were performed on compound eyes from adult specimens of the moths *Celerio euphorbiae* and *Deilephila elpenor* (Sphingidae). A few experiments were also done on *Noctua pronuba* (Noctuidae). The species were identified by the names given in the list of Lepidoptera published by Heslop (1959, 1960).

The specimens of *Noctua pronuba* were caught wild and the Sphingidae were raised in captivity. The experiments on the specimens caught wild were done within 2 days after capture. Moths raised from pupae were used for experiments 1 to 7 days after their emergence. The specimens were usually kept in darkness for 12 hours before the experiments, the minimum time being 4 hours, and the maximum time 24 hours. The experiments were done at room temperature 20 to 26°C. Experiments in which the eyes were illuminated through a glass fiber were done on 310 eyes. These experiments were performed on *Celerio euphorbiae* and *Deilephila elpenor*. All eyes were of the normal type containing dark pigment granules. Experiments were done both at night time and daytime. The results were not influenced by the time of day when the experiments were done.

**Dissection** The eye was excised in such a way that the chitinous ocular ridge or sclerite ring (see Snodgrass 1935 and Yagi and Koyama 1963) remained intact. The optic lobe was then removed except for the lamina (see Bullock and Horridge 1965) and, in some eyes part of the medulla. The parts of the optic lobe that were not removed were severed with a fine needle. The dissection was performed under dim white light. Throughout the dissection the eyes exhibited a maximal glow (page 16). If the glow diminished during the dissection the eye was discarded.

In some experiments the cornea, crystalline cones and distal pigments were removed prior to the electrophysiological measurements. The cornea was cut around the margin of the eye and was removed from the eye together with the crystalline cones and the distal pigments which adhered to the cornea. The dissection was made when the glow was maximal so that the reticular rhabdoms were left intact. Microscopical inspection showed that the distal pigment granules were removed from all parts of the eye except in those ommatidia immediately adjacent to the sclerite.

**Light stimulation** During the electrophysiological experiments the eye was

kept in a light tight box. The adapting and test lights were obtained from a tungsten lamp mounted in a Zeiss dissecting microscope (Zeiss tungsten lamp 390158 operated at 72 V AC from a stabilized power source color temperature 2900 to 3100°K). The unattenuated light intensity measured at the corner was about 35000 lux for both the adapting and test lights. In this paper the relative intensities are given in logarithmic units with the unattenuated intensity as reference level. The unattenuated intensity is referred to as log relative intensity 0 ( $\log_{10} 1$ ), an intensity attenuated to 1/10 is log relative intensity -1 ( $\log_{10} 0.1$ ) etc. The beam of light from the microscope was split into two beams one beam being used as the adapting light and the other as the test light. The area illuminated by the adapting light was identical with that illuminated by the test light. This area was many times larger than the surface of the eye. The intensities of the adapting and test lights were independently controlled by neutral density filters (Bausch and Lomb 31 34 38 or Schott NG 3) and a heat filter (Balzer Calflex B 1 K 1 or B 1 K 2). In addition the intensity of the test light could be gradually attenuated by a neutral density wedge (Kodak). Control experiments showed that the threshold value did not change if, in the course of an experiment the two optical paths were interchanged. The positioning of the eye in the center of the illuminated field was not critical since the eye could be displaced by more than its own diameter without any measurable change in threshold value.

The duration of the test flashes was 0.2 sec. This duration permitted the amplitude of the retinal potential change to be measured, but was short enough not to cause any measurable change in the threshold value. The duration of the test illumination was controlled by a mechanical shutter (Praktika). The exposure time was regularly checked by means of a photocell connected to the oscilloscope. The duration of the adapting illumination was controlled by a manually operated shutter.

Test flashes were also conducted to the eye through a coated glass fiber (American Optical Company core glass refractive index = 1.62 coating glass refractive index = 1.52). The fiber had a total diameter of 75  $\mu\text{m}$  and a total length of about 15 cm. The fiber was inserted into the eye by a micromanipulator the depth being determined by a micrometer gauge. Illumination was provided by a tungsten lamp (Osram 8032 operated at 12 V AC color temperature 2900 to 3000°K or 18 V AC color temperature 3300 to 3400°K. The operating voltage was provided by a stabilized power source). When one end of the glass fiber was exposed to the unattenuated light from the tungsten lamp the luminous flux emitted from the other end of the fiber was 25  $\mu\text{lm}$  (12 V) or 140  $\mu\text{lm}$  (18 V). The maximal luminous intensity was 3  $\mu\text{cd}$  (12 V) or 17  $\mu\text{cd}$  (18 V). The unattenuated light intensity is referred

to as log relative intensity 0. The intensity of the light entering the glass fiber was attenuated by filters and a wedge of the same specifications as mentioned above. The duration of the test flashes, 0.2 sec, was controlled by a mechanical shutter (Ibsor). The spectral transmittance of the fiber was measured in a spectrophotometer (Spectronic). According to these measurements the maximal deviation from perfect neutrality was about  $\pm 20\%$  for wavelengths between 450 and 700 nm. The transmittance was less for wavelengths shorter than 450 nm. Control experiments using fibers that had been given an extra coating of black lacquer showed that the experimental results were not influenced by light escaping through the glass fiber walls.

Figure 1 A shows a photograph of an eye with a fiber penetrating the cornea. Figure 1 B shows a histological section of an eye with a fiber drawn to scale. It is seen that the diameter of the fiber is about 3 times the diameter of an ommatidium. Inspection of sliced fresh eyes showed that in spite of the small diameter of the fiber, most of the ommatidia within the eye were illuminated by the light coming from the fiber. This may partly be due to the fact that the beam of light emitted from the fiber was very divergent. It may also be due to reflection and scatter by retinal structures such as the tracheae and reticular cells.

Figure 2 is a schematic drawing indicating various positions of the fiber tip within the eye. In most experiments the fiber was inserted through the cornea and the tip was placed between the accessory pigment and the pigment near the basement membrane (Fig. 2 A and B). In some experiments the fiber was inserted through the basement membrane and the tip was located proximal to the accessory pigment (Fig. 2 C) and in other experiments the tip was located just below the inner surface of the cornea (Fig. 2 D).

*Recording of potential changes.* The eye was placed on a filter paper moistened with a saline solution (Ephrussi and Beadle 1936). The eye was fixed to a cork plate by means of pins through the chitin surrounding the eye. The recording electrode was an Ag/AgCl wire (diameter 50 or 100  $\mu\text{m}$ ) the tip of which was placed just below the cornea. Another Ag/AgCl wire serving as the reference electrode was placed in contact with the moistened filter paper and connected to ground. Eyes from which the cornea and distal pigments had been removed were placed in a moist chamber during the experiment and the recording electrode was placed in contact with the exposed reticular cells.

The electrodes were connected via a cathode follower to a DC preamplifier (Grass P6 frequency response 0 to 10000 Hz) and a cathode ray oscilloscope (Tektronix 502). In most experiments a capacitor was inserted between the preamplifier and the oscilloscope. The resulting coupling time constant of 10 sec was sufficiently long not to alter significantly the form of the potential

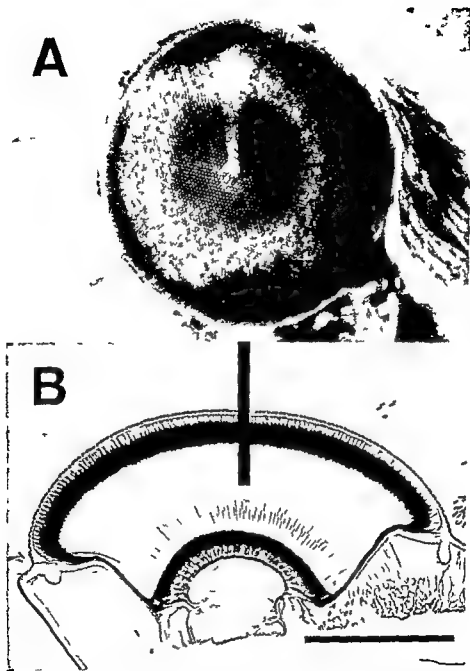


Fig 1 A compound eye of night moth (*Noctua pronuba*) with light conducting glass fiber penetrating through cornea B histological section of eye (*Celerio euphorbia*) showing position of glass fiber Fiber tip located between accessory pigment and pigment near basement membrane Fiber diameter ( $75\ \mu\text{m}$ ) drawn to scale in Fig 1B Horizontal bar 1 mm

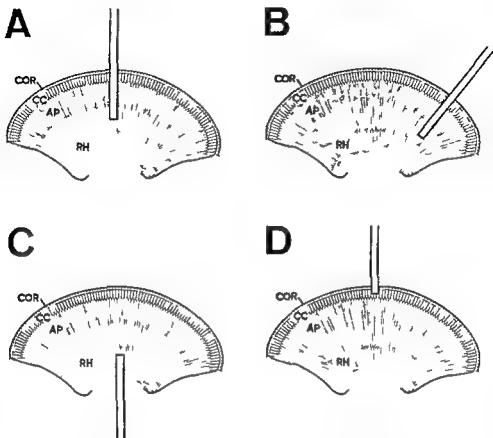


Fig 2 Schematic drawing showing fiber tip at different positions within the eye A fiber inserted through cornea in central part of eye and tip located between accessory pigment and pigment near basement membrane B fiber inserted near margin of eye C fiber inserted through retinal basement membrane D fiber tip located just below inner surface of cornea COR = cornea CC = crystalline cones AP = accessory pigment RH = rhabdoms

change occurring in response to a 0.2 sec light flash. Some experiments were also done with direct coupling.

The electrodes were coated with black lacquer except at the tip. Tests for photoelectric potential changes caused by illumination of the electrodes were performed periodically. No such potential changes were recorded at the light intensities used in this investigation.

**Determination of threshold response** The retinal potential change chosen as threshold response was generally 40 or 60  $\mu\text{V}$  (in some experiments 80  $\mu\text{V}$ ). The relative light intensity eliciting this potential change is referred to as  $T$  when the eye was illuminated in the conventional manner and  $t$  when the eye



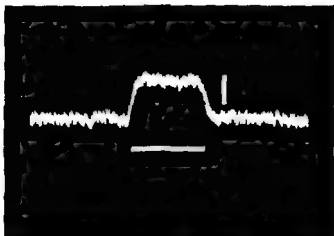


Fig 3 Response of eye to light stimulation of threshold intensity. Vertical bar 50  $\mu$ V. Horizontal bar 200 msec. Upward deflection indicates negativity of retinal electrode.

was illuminated through the glass fiber irrespective of the position of the fiber tip within the eye. Stimulation of the eye by light passing through the ommatidial dioptric media is called transpigment stimulation and the threshold intensity using this stimulation is the transpigment threshold. Stimulation through a fiber the tip of which was located proximal to the accessory pigment is referred to as infrapigment stimulation and the threshold is the infrapigment threshold.

The potential change in response to a 0.2 sec test flash is shown in Fig 3. The electrode inserted through the corner became negative with respect to the reference electrode and the negativity was maintained until the end of the flash. The potential change was the same whether the eye was exposed to transpigment or infrapigment light stimulation. Regardless of the pigment position the amplitude of the initial transient component of the threshold response did not exceed the amplitude of the sustained component by more than about 10 to 15  $\mu$ V at the adapting light intensities used in this study.

In darkness and at the adapting light intensities used in the electrophysiological experiments (not stronger than log relative intensity  $-1$ ) an unattenuated test light usually elicited a response the amplitude of which was at least 20 times larger than the value chosen as the threshold response. The only exception was that when the eye was exposed to an adapting light intensity of log relative intensity  $-1$  with the accessory pigment situated distally the response elicited by the unattenuated test light was usually 2 to 5 times greater than the amplitude chosen as threshold.

In darkness and at the adapting light intensities used in this study a change in transpigment or infrapigment test light intensity by 0.2 log unit caused a change in response amplitude of about 20  $\mu$ V or more. A response of 20  $\mu$ V was usually the smallest response that could be clearly separated from the noise. The accuracy of the measurements of  $T$  and  $t$  was therefore estimated to be about  $\pm 0.2$  log unit. The minimum interval between test flashes was about 0.5 min except in some experiments in which test flashes were applied at 0.1 min intervals during the first minute of exposure to an adapting light. Control experiments showed that at these frequencies the test stimuli did not cause any measurable change in  $T$  or  $t$ .

*Recording of glow* When the eyes were exposed to an adapting light this light was also used for observing the size of the glow (page 16). The glow in eyes that were not exposed to an adapting light was observed under a dim white light for about 5 sec immediately after the threshold determinations. Control experiments showed that this illumination did not change the size of the glow nor did it change the values for  $T$  and  $t$  when tested 0.5 min after the observations of the glow.

Photographs of some eyes were taken with a microscope camera (Zeiss Aufsetz Kamera). The light source was provided by an electronic flash (Zeiss).

*Histological procedures* The eyes were kept in Bouin—Duboscq—Brasil's fixation solution (see e.g. Romeis 1948 or Gatenby and Beams 1950) for 12 to 16 hours. They were then successively transferred into 80 and 96 % ethanol, Bolcek's solution (see e.g. Romeis 1948 or Gatenby and Beams 1950), 96 and 99 % ethanol and a mixture of ethanol and anhydrous ether. The eyes were embedded in celloidin. The unstained sections (about 30  $\mu$ m thick) were mounted in Canada balsam. The position of the pigment borders in the histological sections was measured at  $0^\circ$ ,  $\pm 15^\circ$ ,  $\pm 30^\circ$  and  $\pm 45^\circ$  from the central ommatidia. The pigment position is given as the mean of these 7 measurements.

The following procedure was used to relate glow size to pigment position. Excised dark adapted eyes, usually with part of the optic lobe left intact, were kept in glass containers and the entire corneal surface was inspected at intervals through the optical system used for observation of the glow during the electrophysiological experiments. One series of eyes was kept in darkness. The eyes in which the glow had been maximal (page 16) in all parts of the eye for 15 to 30 min were submerged in Bouin—Duboscq—Brasil's fixation solution and left in darkness. Another series of dark adapted eyes was exposed to weak light for 20 to 30 min. The eyes exhibiting minimal glow (page 16) in all parts of the eye were exposed to the same light for another 20 min. The eyes in which the glow remained minimal were submerged in Bouin—Duboscq—Brasil's fixation solution and kept in the same illumination.

*Measurements on fresh eyes* In one series of experiments the central part of the eye was sliced with a razor blade at right angles to the corneal surface. The eye was placed on a glass cover slip and the distance between the corneal surface and the distal border of the pigment near the basement membrane was measured with an ocular micrometer through a dissecting microscope. The results of these measurements are summarized in Table I. The results of the same measurements from histological sections (also given in Table I) indicate that the histological procedure caused a shrinkage of about 10 to 15% both in *Celerio euphorbiae* and *Deilephila elpenor*. This shrinkage was taken into account when the position of the fiber tip was determined from histological sections.

TABLE I. Distance ( $\mu$ m) between distal surface of cornea and pigment near basement membrane in fresh eyes and in histological sections. Mean values and standard deviation (S.D.). Number of eyes = n.

Species		Fresh eyes	Histological sections
<i>Celerio euphorbiae</i>	Mean	961	844
	S.D.	45	55
	n	32	116
<i>Deilephila elpenor</i>	Mean	868	780
	S.D.	69	47
	n	20	99

*Symbols used in the illustrations* In the illustrations symbols are used which indicate the position of the glass fiber tip within the eye and the size of the glow. It is also indicated in the illustrations whether the eyes were kept in darkness or exposed to an adapting light. The symbols are explained on page 15.



No fiber used



Fiber inserted through cornea and tip located between accessory pigment and pigment near basement membrane



Fiber tip located just below inner surface of cornea



Fiber inserted through retinal basement membrane and tip located between accessory pigment and pigment near basement membrane



Distal pigments removed together with cornea



Maximal glow



Small glow



No glow



Threshold  $T$



Threshold  $t$



Eye kept in darkness



Eye exposed to adapting light



Neutral density filter substituting for pigment

## I Pigment migration and "glow"

It is well known that the compound eyes of nocturnal moths and other arthropods reflect light when the eye is dark adapted. The reflected light is seen as a bright spot (Leydig 1864), usually referred to as the 'glow', in the part of the eye directed towards the observer (see reviews by Parker 1932 and Goldsmith 1964). The light is reflected from structures within the eye in moths probably mainly from the tracheae (Exner 1891, Merker 1929, Yagi and Koyama 1963). However, according to Day (1941) it is uncertain whether or not the tracheae contribute to the reflection. The glow gradually disappears when the eye is illuminated and reappears after the eye is transferred to darkness again.

Several authors (Exner 1891, Kiesel 1894, von Frisch 1908, Demoll 1917, Merker 1929, Horstmann 1935, Höglund 1963 a, Yagi and Koyama 1963) have reported that movements of the accessory pigment are associated with changes in the size of the glow. However, Parker (1932) has pointed out that glow changes in compound eyes may be determined not only by movements of the accessory pigment but also by movements of pigment located proximally in the eye (see also Goldsmith 1964), and Day (1941) reported that in the compound eye of the moth *Ephesia* the glow rapidly disappears with almost imperceptible changes in the position of the accessory pigment cells. Changes in the size of the glow may therefore be associated with movements of the accessory pigment in some species but not in others.

This section of the paper reports experiments undertaken to study whether or not glow changes may be used as an index of pigment movements in *Celerio euphorbiae*, *Deilephila elpenor* and *Noctua pronuba*.

The anatomy of the eye in *Celerio euphorbiae* has been described by Bugmon and Popoff (1914) and the pigment movements by Stefanowska (1890). A recent general description of the compound eye in the Sphingidae and Noctuidae is given by Yagi and Koyama (1963).

## RESULTS

Figure 4 shows a compound eye with no glow (A), with a small glow (B), and with a maximal glow (C). The typical pigment position in an eye from *Celerio*

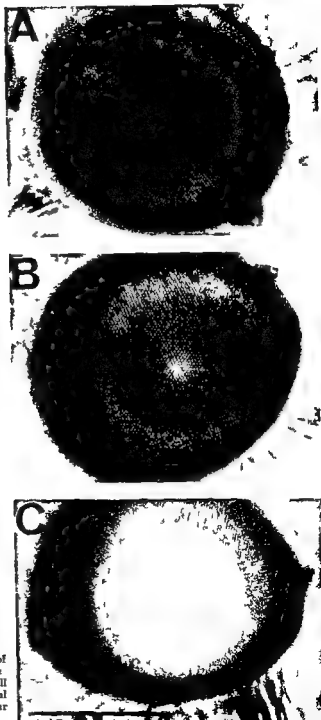


Fig. 4 Compound eye of night moth (*Noctua pronuba*) with no glow (A) small glow (B) and maximal glow (C) Horizontal bar 1 mm

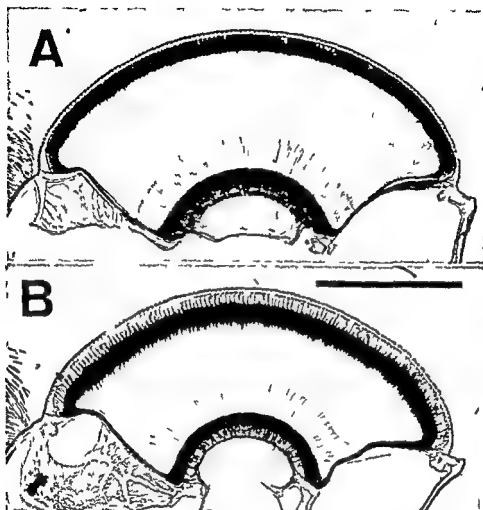


FIG. 5. Position of light screening accessory pigment in eyes (*Celerio euphorbiae*) exhibiting maximal glow (A) and minimal glow (B). Note that in eye with minimal glow pigment is located more proximally than in eye with maximal glow. Horizontal bar 1 mm.

or *Deslephula* with a maximal glow is illustrated in Fig. 5 A. The accessory pigment is situated around the crystalline cones with the distal pigment border located immediately below the inner corneal surface, and the proximal border almost at the level of the proximal ends of the crystalline cones. Figure 5 B illustrates the typical pigment position in an eye exhibiting a minimal glow, i.e. a glow which is just visible as a minute spot in the center of the area under inspection. The distal pigment border is seen to surround the crystalline cones, and the proximal border is located around the distal part of the reticular cytoplasmic processes.

Values for the distances between the corneal surface and the distal and proximal borders of the accessory pigment in eyes from *Celerio euphorbiae* and *Deilephila elpenor* with maximal and minimal glow are given in Table II. A statistical 'rank sum test' (see e.g. Dixon and Massey 1957) showed a highly significant difference ( $p < 0.001$ ) between the pigment position in eyes exhibiting maximal and minimal glow. Sections were also made of eyes from *Noctua pronuba*. In this species, as in *Celerio* and *Deilephila*, the pigment was situated more proximally in eyes with minimal glow compared to eyes with maximal glow. The results show that in the species investigated a diminution of the glow from maximal to minimal is associated with migration of the accessory pigment in the proximal direction and *vice versa* (see also Hoglund 1963 a).

TABLE II Distance ( $\mu\text{m}$ ) from proximal surface of cornea to proximal and distal borders of accessory pigment in histological sections of eyes exhibiting maximal or minimal glow. Median values and range. Number of eyes = n.

Species	Glow size		Proximal surface of cornea to		
			Distal pigment border	Proximal pigment border	Distal end of rhabdoms
<i>Celerio euphorbiae</i>	Maximal	Median Range n	0 0—19 27	132 103—190 22	611 499—727 27
	Minimal	Median Range n	38 0—106 94	219 136—297 94	599 461—724 94
<i>Deilephila elpenor</i>	Maximal	Median Range n	0 0—13 26	133 89—165 26	555 500—600 26
	Minimal	Median Range n	74 9—139 73	223 141—290 73	576 441—648 73

It should be noted that in eyes that have been adapted to a bright light both the proximal and distal borders of the accessory pigment are located considerably more proximally than in eyes exhibiting a minimal glow (see Fig. 13 D, page 35).

In some species of night moths the position of the pigment located near the basement membrane varies with the adapting light intensity (Tuurala 1954). In these eyes the pigment migrates in the proximal direction in darkness, and in the distal direction when the eye is illuminated. Such pigment movements



have been reported to occur in *Celerio euphorbiae* (Stefanowski 1890). The magnitude of the pigment movements was estimated by measurements of the pigment position in dark adapted eyes and in eyes that had been exposed to the unattenuated adapting light (log relative intensity 0) for more than 30 min. The mean distance between the basement membrane and the distal border of the pigment in dark adapted and light adapted eyes is given in Table III. The results show that movements of the pigment near the basement membrane if they occur at all do not exceed a few  $\mu\text{m}$  at the light intensities used in this study.

TABLE III Distance ( $\mu\text{m}$ ) from basement membrane to distal border of pigment near basement membrane in histological sections of dark adapted and light adapted eyes. Mean values and standard deviation (S.D.). Number of eyes = n.

Species		Dark adapted	Light adapted
<i>Celerio euphorbiae</i>	Mean	19	20
	S.D.	1.4	1.9
	n	14	19
<i>Deilephila elpenor</i>	Mean	16	17
	S.D.	1.5	1.1
	n	15	20

## DISCUSSION

The present results are in agreement with the conclusions reached by most earlier investigators (Exner 1891, Kiesel 1894, von Frisch 1908, Demoll 1917, Merker 1929, Horstmann 1935, Yagi and Koyama 1963) that the size of the glow is related to the position of the accessory pigment but are not in accordance with the observation by Day (1941) in experiments on *Ephesia* that the glow may disappear with almost imperceptible changes in position of the pigment cells. This discrepancy indicates that apart from the possibility of species variation the results may be influenced by the experimental technique. Some earlier investigators have used variations in the size of the glow as an index of accessory pigment movements without correlating the size of the glow to the position of the pigment in histological sections. However, Kiesel (1894) and Horstmann (1935) reported that the glow disappears before the pigment has reached the extreme proximal position. Yagi and Koyama (1963) reported that the diminution of the glow occurs when the accessory pigment passes the proximal end of the crystalline cones. A schematic illustration shows a minute

glow spot when the pigment is surrounding the proximal part of the crystalline cone. The present results are consistent with those described by Yagi and Koyama.

It is unlikely that movements of the pigment near the basement membrane a few  $\mu\text{m}$  could significantly change the reflection of light by the tracheae and thereby cause any variation in glow, since the tracheae extend almost to the proximal end of the rhabdom (Bugnon and Popoff 1914; Yagi and Koyama 1963). The main cause for the glow changes seems to be variations in the light screening by the accessory pigment (Exner 1891).

Edwards (1964) has suggested that glow changes observed in pigment free eyes of the moth *Halimodota* are caused by movements of the accessory pigment cell nuclei. Even if movements of these nuclei contribute to the glow changes also in normal eyes, the present experiments show that variations in glow are associated with movements of the accessory pigment. It should be noted, however, that the glow disappears before the pigment has moved to its most proximal position. Pigment movements can therefore occur in the absence of glow. The reticular cell nuclei are not mobile in the Sphingidae (Yagi and Koyama 1963) and should not contribute to glow variations in *Celerio* and *Deilephila*.

## II Pigment migration and threshold changes in dark-adapted eyes kept in darkness

Exner (1891) suggested that in the compound eye the light attenuation varies with the pigment position. According to this hypothesis the outward pigment movement causes a gradual increase in the light transmission to the reticular rhabdomeres. The outward pigment migration occurring during dark adaptation in the compound eye of moths is associated with a fall in (transpigment) threshold of 2 to 3 log units (Bernhard and Ottoson 1960 a 1960 b 1964, Bernhard, Hoglund and Ottoson 1963, Post and Goldsmith 1965). The relation to the pigment movement suggests that the threshold decrease is caused by a decreased screening effect of the migrating pigment. This assumption is supported by the observation that the second phase of the threshold decrease is absent if the pigment fails to migrate (Bernhard and Ottoson 1960 b), and also by the results of behavioral experiments on the moth *Ephestia* (Klingebeil 1938) and electrophysiological experiments on *Calliphora* (Autrum 1955) showing that eyes lacking dark pigment are more light sensitive than normal eyes.

The experiments in this section investigate the effect of the pigment migration on the light transmission and on the light sensitivity of the receptors. In relation to this problem Smith and Kümeldorf (1964) found, in experiments on the moths *Agrotis* and *Pseudaletia*, that after the end of an adapting light exposure the response to  $\beta$  radiation reached a maximal amplitude many minutes before the response to light reached its maximum. They concluded that  $\beta$  radiation is not attenuated by the pigment, whereas the slower increase in amplitude in response to light stimulation is due to the pigment migration. On the other hand Baldwin and Sutherland (1965) reported that the time course of the increase in the amplitude of the response to  $\lambda$  ray test flashes during dark adaptation in the cockroach *Blaberus* appears to follow pigment migration.

The eyes used in the experiments described in this section had been kept in darkness for several hours before the experiments were started. A maximal glow in all parts of the eyes showed that the pigment was located distally when the determinations of  $T$  and  $t$  (page 11) began. The eyes were kept in darkness throughout the experiments and pigment movements were determined by observations of changes in glow size and by histological sections.

## RESULTS

In some eyes the glow remained maximal as long as the experiments were continued indicating that the pigment remained in the extreme distal position. The maximal duration of such an experiment was 4 hours. Histological sections confirmed that the pigment was located distally in all parts of the eye. Figure 6 illustrates that both  $T$  and  $t$  remained constant throughout the experiments. The variations in threshold did not exceed 0.2 log unit which corresponds to the accuracy of the threshold measurements.

In most eyes the glow gradually diminished after the experiment was started and then disappeared indicating that the pigment had migrated in the proximal direction. Such an experiment is shown in Fig. 7. During the first 25 min when the glow was maximal both  $T$  and  $t$  remained constant. The glow then

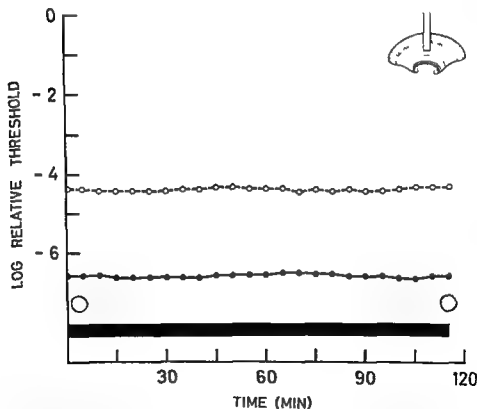


Fig. 6.  $T$  and  $t$  in dark adapted eye (*Deilephila elenor*) kept in darkness. Pigment in extreme distal position. Both  $T$  and  $t$  were constant throughout the experiment. (For explanation of symbols see page 15)

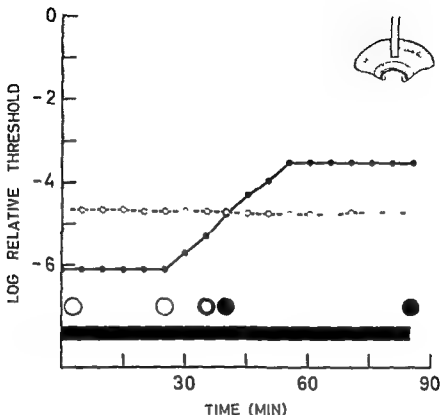


Fig 7  $T$  and  $t$  during inward pigment migration in dark adapted eye (*Desiphrula eipenor*) kept in darkness. Note that pigment migration was associated with increase in  $T$  while  $t$  remained constant. (For explanation of symbols see page 15)

began to diminish and simultaneously  $T$  began to increase. The glow disappeared after 10 min. The total increase in  $T$  was 2.6 log units, after which  $T$  remained constant until the experiment was terminated. The glow did not reappear. No glow was seen in any part of the eye at the end of the experiment indicating that the pigment had migrated inwards in all parts of the eye. This was confirmed in histological sections which also showed that the fiber tip had been placed below the accessory pigment during the experiment. As seen in Fig 7,  $t$  remained constant throughout the experiment in spite of the pigment migration.

In this series of experiments the interval between the start of the experiment and the onset of the diminution of the glow varied from a few minutes in some experiments to about 2 hours in others. Irrespective of the duration of this interval  $T$  remained constant as long as the glow was maximal and increased

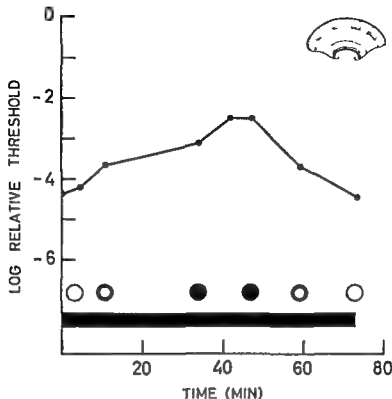


Fig 8  $T$  during inward and outward pigment migration in dark adapted eye (*Noctua pronuba*) kept in darkness. Note increase in  $T$  during inward pigment movement and decrease during outward movement. (For explanation of symbols see page 15)

when the glow diminished while  $t$  remained constant even when the pigment migrated. The maximal increase in  $T$  was about 2.6 log units.

During the experiments the glow could be observed only within a restricted part of the eye. It is well known that the pigment can migrate in some ommatidia while remaining stationary in other ommatidia (Exner 1891, Day 1941, Bernhard 1963, Yagi and Koyama 1963). Immediately after each experiment, the entire surface was therefore inspected. In most experiments the glow had disappeared in all parts of the eye. The only exception was found in experiments terminated shortly after the glow had disappeared in the part of the eye observed during the determinations of  $T$  and  $t$ . In some of these eyes the glow could still be seen in other parts of the eye. However, in experiments like the one shown in Fig 7, in which the increase in  $T$  had reached a steady maximal value, the glow had disappeared in all parts of the eye.

The observation that  $t$  remained constant during the inward pigment move

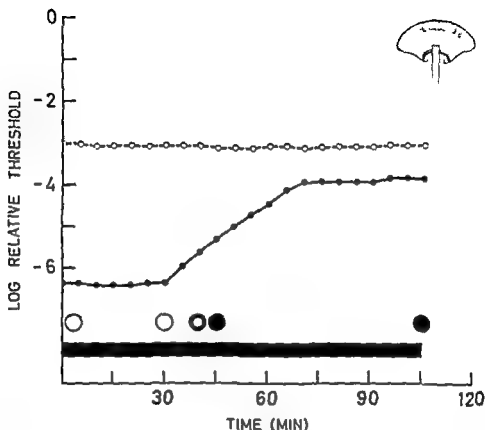


Fig 9  $T$  and  $t$  during inward pigment migration in dark adapted eye (*Deilephila elpenor*) kept in darkness. Fiber inserted through basement membrane and tip located proximal to accessory pigment (For explanation of symbols see page 15)

ment suggests that the receptor light sensitivity remained constant. The increase in  $T$  would thus be caused by an increase in light attenuation due to the pigment movement.

If this interpretation is correct  $T$  would decrease in eyes where the inward pigment movement is followed by an outward movement. That this is so is illustrated in Fig 11. In this experiment  $T$  increased when the glow diminished and disappeared. The reappearance and increase of the glow was accompanied by a decrease in  $T$ . If the retinal sensitivity remains constant when the pigment migrates  $t$  should remain constant regardless of the position of the fiber tip between the accessory pigment and the pigment near the basement membrane. Experiments were done with the tip placed at different positions between these pigment layers. The fiber was introduced near the margins as well as in the central parts of the eyes. The depth to which the fiber tip was advanced

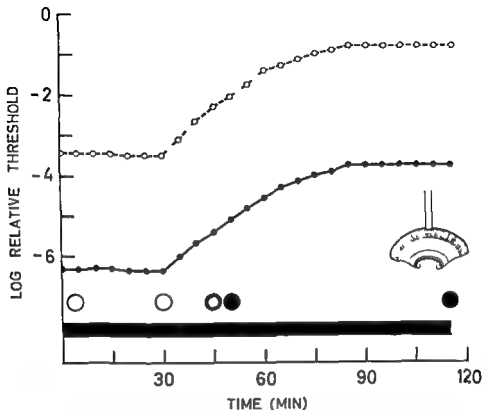


Fig 10  $T$  and  $t$  during inward pigment migration in dark adapted eye (*Celerio euphorbiae*) kept in darkness. Fiber tip located just below inner surface of cornea. Note increase in both  $T$  and  $t$  during pigment migration (For explanation of symbols see page 15)

also varied in some eyes the tip was located near the basement membrane and in other eyes above the rhabdoms. In some eyes the fiber was introduced through the basement membrane and the tip placed proximal to the accessory pigment. Such an experiment is shown in Fig 9. In this experiment  $t$  was somewhat higher than in the experiment shown in Fig 7, which may be due to the fact that in one eye the tip pointed towards the accessory pigment (Fig 9) whereas in the other the tip pointed towards the light sensitive rhabdomeres (Fig 7). In all of these experiments  $t$  remained constant when the accessory pigment migrated irrespective of where the tip was located between the accessory pigment and the pigment near the basement membrane.

In some experiments the fiber tip was placed just below the cornea so that the test light from the fiber passed through the accessory pigment before reaching the rhabdomeres (Fig 10). In these experiments when the pigment started



to migrate inwards, both  $T$  and  $t$  began to increase and continued to increase until they simultaneously reached their maximal values

In dark adapted eyes from which the distal pigments were removed (page 7)  $T$  and  $t$  usually remained constant as long as the experiment was continued. In a few experiments both  $T$  and  $t$  slowly increased, presumably due to deterioration of the preparation. In such experiments there was a simultaneous change in both thresholds.

## DISCUSSION

There is considerable evidence to show that the extracellularly recorded maintained corneal negative response obtained after removal or severance of the optic lobe mainly arises in the reticular cells (Bernhard 1942, Jahn and Wulff 1942, Autrum and Gallwitz 1951, Hartline, Wagner and Mac Nichol 1952, Autrum and Hoffmann 1960, Autrum, Autrum and Hoffmann 1961, Goldsmith 1960, Ruck 1961). It is therefore probable that the electrical responses recorded in this study mainly originated in these cells. However, according to Svastichun, Fernandez Moran and Jonasson (1956) a potential change can be recorded when an isolated corneal pigment cell preparation from moths such as *Erebos* is exposed to light. The maximal amplitude of the response recorded from this preparation was 6 mV, and the authors concluded that the potential was generated in the region of the pigment cell and crystalline cone layer. The question then arises whether the changes in transpigment threshold during the pigment migration were caused by a change in the threshold of an electrical response from this layer. When the fiber tip was placed just below the cornea  $t$  varied with the pigment position and when the tip was placed below the accessory pigment  $t$  remained constant during pigment movements. In both these experiments the light from the fiber illuminated the pigment cells. It seems probable that  $t$  should have changed in both experiments if the pigment movements were accompanied by a change in electrical response from the pigment cell and crystalline cone layer sufficiently large to influence the threshold measurements. It is therefore unlikely that the changes in transpigment threshold were due to variations in an electrical response from the pigment cell and crystalline cone layer.

It may be asked whether the threshold changes were caused by variations in light screening by structures proximal to the accessory pigment. Since movements of the pigment near the basement membrane do not exceed a few  $\mu\text{m}$  at the intensities used in this study it is not likely that they could alter the light screening effect sufficiently to cause a change in threshold. Furthermore, if the

variations in transpigment threshold were caused by photomechanical changes proximal to the accessory pigment one would expect a change not only in the transpigment threshold but also in the infrapigment threshold. It is therefore improbable that photomechanical changes in structures proximal to the accessory pigment contributed to the change in transpigment threshold.

Movements not only of the accessory pigment granules but also of the pigment cell nuclei may contribute to the change in screening effect. This distinction is made with reference to the observation by Edwards (1964) that movements of these nuclei may cause glow changes in eyes devoid of pigment. The reticular cell nuclei do not migrate in the Spalangidae (Yagi and Koyama 1963) and therefore should not contribute to the changes in light transmission in *Celerio* and *Deilephila*.

The experimental results suggest that the decrease in (transpigment) threshold associated with the outward pigment migration during dark adaptation (Bernhard and Ottoson 1960 a, 1960 b; Bernhard, Hoglund and Ottoson 1963; Post and Goldsmith 1965) is caused by a decrease in the light attenuation by the pigment. This conclusion is supported by infrapigment threshold measurements showing that after cessation of the adapting light the photoreceptors dark adapt completely even if the pigment fails to migrate distally (page 38).

The results described here are consistent with the conclusion reached by Smith and Karmelndorf (1964) that the pigment migration during dark adaptation causes a decrease in light attenuation by the accessory pigment. Baldwin and Sutherland (1965) did not specify the extent of the pigment migration in their experiments on *Blaberus*. However, their report that the increase in response amplitude to X-ray stimulation appears to follow pigment migration does not exclude the possibility that the light transmission varies with the pigment position.

The increase in light attenuation caused by the inward pigment movement may not be identical to attenuation by a neutral density filter. Wave lengths in the red region of the spectrum pass through the screening pigment in *Calliphora* and *Musca* (Autrum 1955; Autrum and Burkhardt 1961; Hoffmann and Langer 1961; Burkhardt 1962; Goldsmith 1965; Langer and Hoffmann 1966). In the color blind ventral eye of *Notonecta* the spectral efficiency changes during dark adaptation, possibly because red light passes through the pigment (Ludtke 1954; see also Burkhardt 1964). In flies and in *Notonecta* the screening pigment is located between the rhabdoms (see e.g. Goldsmith 1964 or Bullock and Horridge 1965) whereas in the moths used in the present study it is located distal to the rhabdoms. Due to the structural differences between the eyes the effect of the screening pigment in moths may be different from that in flies and in *Notonecta*. The observations on flies and *Notonecta* suggest, however, that in

the compound eye of moths the pigment attenuates some wave lengths more than others, and that the pigment movement thereby causes a change in the spectral energy distribution of the light reaching the rhabdomeres

Pigment movements are usually induced by changes in adapting light intensity, but it is well known that movements can occur also in darkness. A diurnal rhythm in the pigment position has been found in some studies on moths (Kiesel 1894, Demoll 1911, Horstmann 1935, Yagi and Koyama 1963), although in other studies no rhythmic movements were found (Collins 1934, Day 1941). It seems unlikely that the pigment movements occurring in the present experiments were due to diurnal rhythms since the pigment migrated in experiments performed during the night as well as in daytime. The observation that the pigment movement took place within 2 hours, and in some eyes even within a few minutes after the experiments were started suggests that the migration should be attributed to the eyes being isolated from the rest of the body. Isolation of the eye reduces the supply of oxygen and probably disturbs the normal metabolic activity of the retina. In the eye of *Calliphora* the metabolic rate depends on the supply of oxygen to the retina (Langer 1962), but the electrical response from the receptor cells is very resistant to low oxygen tension (Hoffmann 1960). Earlier experimental results on Lepidoptera (Day 1941, Yagi and Koyama 1963 see Goldsmith 1964) and crustaceans (Bennet and Merrick 1932) suggest that a normal metabolic activity is required to keep the pigment in the extreme distal position.

### III Pigment migration and threshold changes during and after illumination of the eye

The time course of the change in (trans)pigment threshold during light adaptation in the compound eye of the moth *Galleria* has recently been investigated by Post and Goldsmith (1965). They found that in most eyes the threshold changes were completed within seconds after the onset of the adapting light and then remained constant in spite of the subsequent inward migration of the accessory pigment. However, in some eyes the threshold slowly increased during the pigment movement. The authors concluded that the threshold increased because the increase in receptor sensitivity did not fully compensate for the increase in light attenuation when the pigment migrated proximally.

A slow potential change possibly related to pigment movements has been found in the stick insect *Carausius* (Schneider 1964). In this species the amplitude of the response to (trans)pigment light stimulation of constant intensity rapidly decreases during the first minute of the illumination. The amplitude then remains at a constant level or in some eyes increases, for a few minutes and then slowly decreases again during about 10 to 30 min. Schneider suggested that the late slow decrease in amplitude is caused by pigment migration.

In the experiments to be described below  $T$  and  $t$  were determined during and after exposing the eyes to an adapting light. In some experiments the adapting illumination was either weak enough or brief enough that it did not induce a pigment movement while in other experiments the intensity and duration of the adapting illumination were sufficient to induce an inward pigment movement.

### RESULTS

Both  $T$  and  $t$  rapidly increased when the eye was exposed to the adapting light, and at all adapting light intensities the increases in  $T$  and  $t$  were completed simultaneously in less than one minute. In some experiments the difference between the increase in  $T$  and that of  $t$  was about 0.2 log unit or less. In other experiments the difference was larger. In such experiments  $t$  usually increased more than  $T$ . The difference may to some extent be attributed to the inaccuracy of the threshold determinations. However, it was mostly found when the fiber tip was placed close to the pigment near the basement membrane or close to

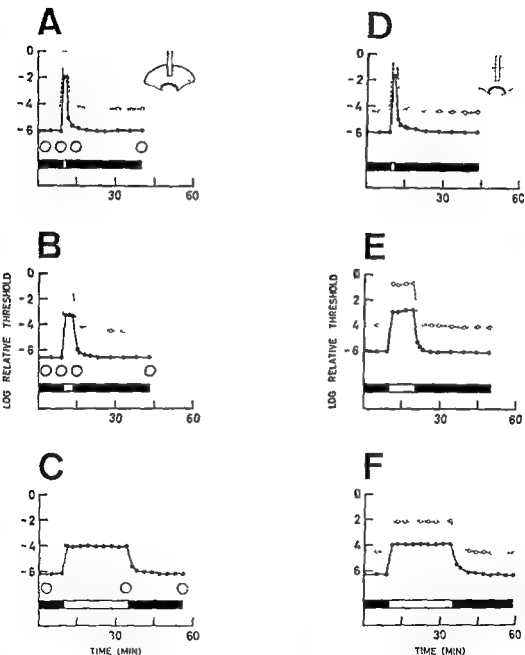


Fig. 11  $T$  and  $t$  before, during and after illumination (A, C, D and F *Delephila eleonorae*; B and E *Celerio euphorbiae*). A—C pigment in distal position during illumination; D—F pigment removed. Log relative adapting light intensity in A and D  $-1$ ; in B and E  $-2$ ; in C and F  $-3$ . Note that  $T$  and  $t$  changed almost in parallel during and after illumination. Experiments made on six eyes. (For explanation of symbols see page 15)

the margin of the eye. These were positions at which the test light from the fiber was likely to illuminate a restricted number of ommatidia. For this reason the fiber tip was usually placed centrally in the eye and usually at least 100  $\mu\text{m}$  from the pigment near the basement membrane.

*Pigment remaining in extreme distal position or pigment removed.* At all intensities the glow remained maximal for some time after the onset of the adapting light. Histological sections showed that as long as the glow remained maximal the pigment was situated distally in all parts of the eye. In one series of experiments dark adapted eyes were exposed to light which was turned off while the glow was still maximal. The changes in  $T$  and  $t$  during 3 such experiments are shown in Fig. 11 A, B and C. Three different light intensities were used. At the highest intensity (log relative intensity  $-1$ , Fig. 11 A) the illumination lasted only for about 2 min, since control experiments showed that with longer exposures the accessory pigment migrated proximally. When log relative intensities  $-2$  and  $-3$  were used (Fig. 11 B and C) the eyes could be illuminated for a longer period before the glow began to diminish.  $T$  and  $t$  rapidly increased at the onset of the adapting light and then remained almost constant as long as the eye was illuminated. When the adapting light was turned off  $T$  and  $t$  gradually decreased to the values obtained before the illumination. The time course of the threshold change found in these experiments was similar to that found for the bee (Goldsmith 1963) in which no pigment movements occur.

Experiments were also made on eyes from which the distal pigments had been removed. Figure 11 D, E and F shows that the time course of the changes in  $T$  and  $t$  during and after illumination closely followed that of the intact eye (Fig. 11 A, B and C). It should be added that  $T$  and  $t$  remained almost constant even when the duration of the adapting light exposure was so long that in the intact eye the accessory pigment would have migrated proximally. However, after the eyes had been exposed to an adapting light of high intensity for many minutes (e.g. log relative intensity  $-1$  for more than 10 min)  $T$  and  $t$  decreased but to values higher than those found before the illumination, which suggests that the preparation had deteriorated during the adapting light exposure.

*Pigment migrating during the illumination.* Figure 12 shows the changes in  $T$  and  $t$  when the adapting light induced an inward migration of the accessory pigment. The glass fiber tip was situated close to the distal part of the rhabdoms, i.e. well below the proximal border of the accessory pigment even when the pigment was in its most proximal position. The eye was kept in darkness at the beginning of the experiment and a maximal glow indicated that the pigment was situated distally. When the eye was illuminated (log relative intensity  $-1$ )  $T$  and  $t$  increased. The increase was completed about 0.5 min

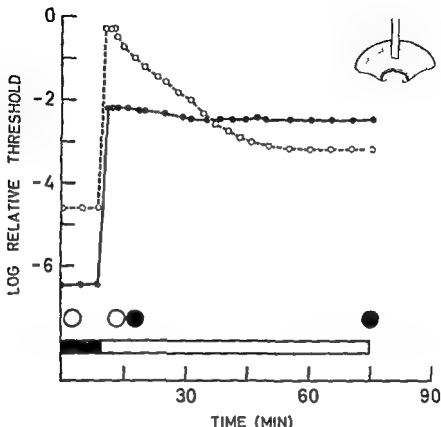


Fig 12  $T$  and  $t$  during illumination (*Deilephila elpenor*). Pigment migrated in proximal direction during illumination. Note that pigment movement was associated with decrease in  $t$  while  $T$  remained almost constant. (For explanation of symbols see page 15.)

after the onset of the adapting light after which  $T$  and  $t$  were almost constant. The glow began to diminish 3 min after the onset of the adapting light indicating that the accessory pigment was migrating proximally. At the same time  $t$  began to decrease. The glow disappeared 8 min after the onset of illumination when  $t$  had decreased by 0.7 log unit. The decrease in  $t$  continued until the eye had been illuminated for 45 min after which  $t$  was constant as long as the eye remained illuminated. The experiment was terminated when the eye had been exposed to the adapting light for 65 min. The total decrease in  $t$  was 2.9 log units. In contrast to the considerable decrease in  $t$  Fig 12 shows that  $T$  decreased only 0.3 log unit.

In all experiments  $t$  was almost constant during the illumination as long as the glow was maximal and decreased when the glow diminished. The change in  $T$  was small compared to that of  $t$ . Histological sections made after the

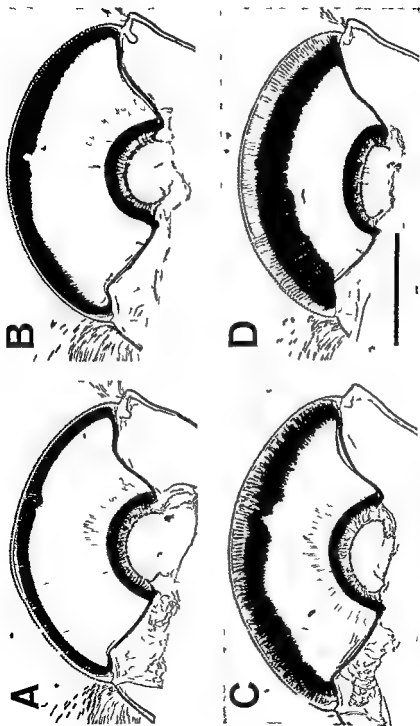


Fig 13 Histological sections of eyes (*Gastero euphorbiae*) showing pigment position after decrease in  $t$  during illumination. Threshold decrease in A 0 log unit in B 11 log unit in C 211 g units in D 30 log units Horizontal bar 1 mm



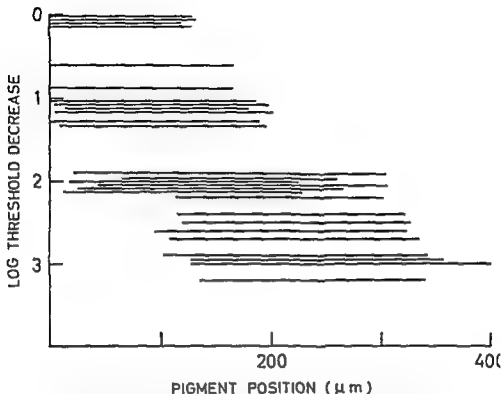


Fig 14 Relation between pigment position and decrease in  $t$  during illumination (*Ce-lerio euphorbiae*). Values from 27 experiments. Ordinate decrease in  $t$ . Abscissa position of accessory pigment in  $\mu\text{m}$  from inner corneal surface. Each horizontal line shows location in one eye. Left end of bar shows position of distal pigment border and right end of bar shows position of proximal border.

decrease in  $t$  was completed, showed that the accessory pigment was situated proximally in all parts of the eyes.

The decrease in  $t$  was correlated with the pigment position in histological sections. Figure 13 A is from an experiment which was terminated before the decrease in  $t$  had begun, and while the glow was still maximal. The accessory pigment is situated in the extreme distal position. Figure 13 B shows the pigment position in an eye when  $t$  had decreased 1.1 log unit; the accessory pigment is situated more proximally. The sections shown in Figs 13 C and D are from eyes used in experiments terminated when  $t$  had decreased 2.1 and 3.0 log units respectively. In these eyes the accessory pigment extends still further proximally. Figure 14 illustrates the relation between the change in  $t$  and the proximal migration of the accessory pigment in 27 experiments. The horizontal lines represent the location of the accessory pigment expressed in  $\mu\text{m}$  from

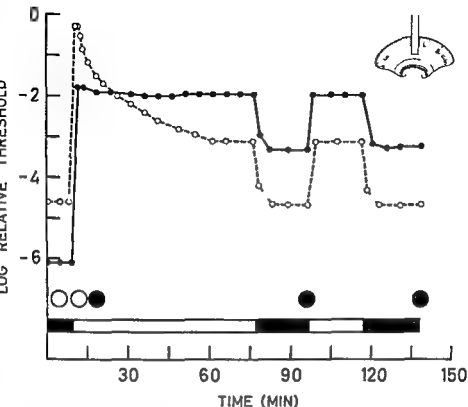


Fig. 15  $T$  and  $t$  before, during and after illumination (*Drosophila elenor*).  $T$  remained almost constant during pigment migration in first illumination period. (For explanation of symbols see page 15)

the inner surface of the cornea. The uppermost lines show the position of the pigment in eyes before  $t$  had started to decrease. It is seen that the gradual expansion and proximal migration of the pigment was accompanied by a gradual decrease in  $t$ .

The relationship between the decrease in the infrapigment threshold and the pigment movement is interpreted as follows. As the pigment migrates proximally the intensity of the light reaching the rhabdomeres is gradually decreased even though the adapting light intensity is constant. The decrease in the infrapigment threshold therefore represents a gradual adaptation of the receptors to the decreasing stimulus intensity, and because of this increase in receptor sensitivity the transpigment threshold remains almost constant in spite of the pigment migration.

Further experiments have provided additional support for this interpretation. In the experiment shown in Fig. 15  $t$  decreased about 2.8 log units while  $T$

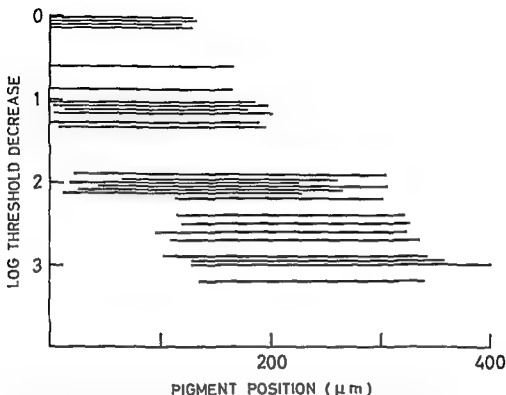


Fig 14 Relation between pigment position and decrease in  $t$  during illumination (*Cerato euphorbiae*). Values from 27 experiments. Ordinate decrease in  $t$ . Abscissa position of accessory pigment in  $\mu\text{m}$  from inner corneal surface. Each horizontal line shows location in one eye. Left end of bar shows position of distal pigment border and right end of bar shows position of proximal border.

decrease in  $t$  was completed, showed that the accessory pigment was situated proximally in all parts of the eyes.

The decrease in  $t$  was correlated with the pigment position in histological sections. Figure 13 A is from an experiment which was terminated before the decrease in  $t$  had begun, and while the glow was still maximal. The accessory pigment is situated in the extreme distal position. Figure 13 B shows the pigment position in an eye when  $t$  had decreased 1.1 log unit, the accessory pigment is situated more proximally. The sections shown in Figs 13 C and D are from eyes used in experiments terminated when  $t$  had decreased 2.1 and 3.0 log units respectively. In these eyes the accessory pigment extends still further proximally. Figure 14 illustrates the relation between the change in  $t$  and the proximal migration of the accessory pigment in 27 experiments. The horizontal lines represent the location of the accessory pigment expressed in  $\mu\text{m}$  from

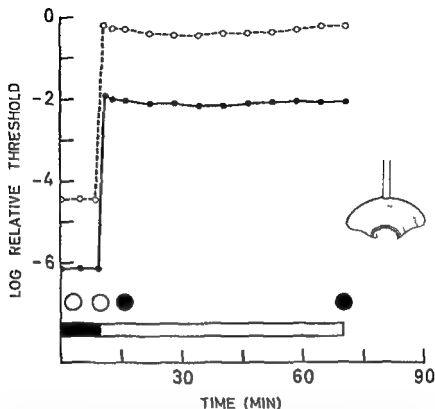


Fig 17 Fiber tip placed just below cornea whereby  $t$  was measured by light passing through pigment layer (*Celerio euphorbiae*). Note absence of decrease in  $t$  during inward pigment movement in illuminated eye (For explanation of symbols see page 15)

**pigment migration** The maximal increase in light attenuation was about 3 log units in this series of experiments. In the experiment shown in Fig 15 the pigment movement caused an increase in light attenuation of about 2.8 log units which was compensated for by the increase in receptor sensitivity so that  $T$  remained almost constant during the pigment migration.

One may predict from the preceding experiments that the increase in receptor sensitivity represented by the decrease in the infrapigment threshold should be related to the increase in light attenuation by the pigment at any time during the adapting illumination. That this is the case was shown in experiments in which the adapting light was turned off while  $t$  was still decreasing. In the experiment shown in Fig 16 the adapting light was turned off at two different times while  $t$  was still decreasing. The decrease in  $t$  was completed during the third light period. In each dark period following these three light periods the final value for  $T$  was higher than during the preceding dark period.  $T$  was

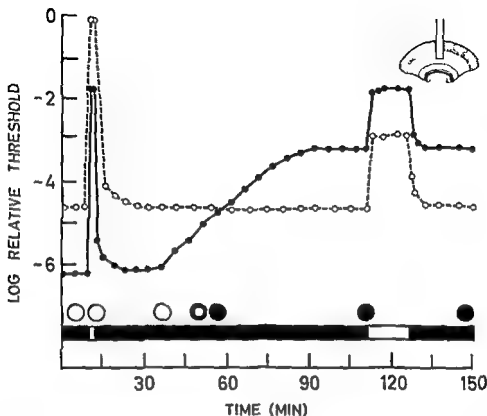


Fig 18  $T$  and  $t$  when pigment migrated in darkness between illumination periods (*Crinia euphorbiae*). During second illumination period  $t$  lower than during first illumination period.  $T$  almost equal during both illumination periods. (For explanation of symbols see page 15)

the same before and after the fourth light period during which there was no decrease in  $t$ . Histological sections of the eye showed that the pigment was located proximally at the end of the experiment.

It might also be expected that if the decrease in  $t$  when the pigment migrates during the illumination represents an increase in receptor sensitivity,  $t$  should decrease only when the fiber tip is placed below the pigment. Figure 17 shows that  $t$  did not decrease when the fiber tip was placed just below the cornea. When the tip was in this position the test light passed through the pigment before it reached the rhabdomeres, and therefore both  $t$  and  $T$  were trans pigment thresholds.

*Pigment migrating during the dark interval between the illumination periods.* Figures 15 and 16 show that  $T$  rapidly decreased during the dark periods and then remained constant as long as the eye was kept in darkness. The diagrams

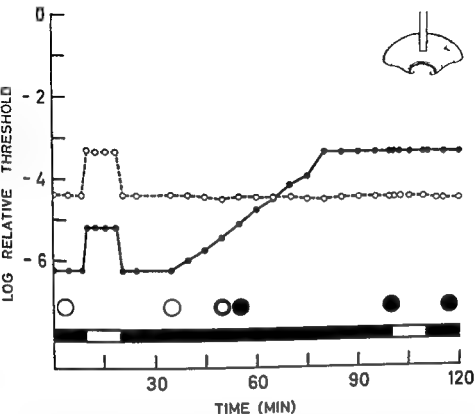


Fig 19  $T$  and  $t$  when pigment migrated in darkness between illumination periods (*Des lephila elphenor*). During second illumination period  $t$  same as in dark adapted eye  $T$  higher during second compared to first illumination period (For explanation of symbols see page 15)

also show that at the beginning of each illumination period  $t$  increased to the same value as that obtained at the end of the preceeding illumination period. It can therefore be concluded that in these as in most other experiments, the proximal pigment migration stopped when the adapting light was turned off and that the pigment remained stationary as long as the eye was in darkness.

In some eyes however the pigment migrated during the dark interval between two illumination periods. In the experiment shown in Fig 18 the adapting light was turned off while the accessory pigment was still in the extreme distal position as evidenced by a maximal glow. Since the pigment had not migrated inwards both  $T$  and  $t$  decreased to the same values as before the illumination. The glow remained maximal for 25 min after which it began to diminish signalling that the accessory pigment was migrating proximally. During the pigment migration  $T$  increased about 2.9 log units and then remained

constant. When the eye was again exposed to the adapting light  $t$  increased to a value that was about 2.8 log units lower than during the preceding illumination period. This experiment shows that the infrapigment threshold depends on the screening effect by the pigment irrespective of whether the pigment migrates during illumination or in darkness.

If the light attenuation by the pigment increases to such an extent that the infrapigment threshold in the illuminated eye is about the same as that of the dark adapted eye, it is to be expected that the value of the transpigment threshold also remains the same as in the dark adapted eye. This transpigment threshold value may be higher than that observed in the illuminated eye with the pigment in the distal position. Such an experiment is shown in Fig. 19. The pigment was located distally at the beginning of the experiment. When the eye was exposed to the adapting light (log relative intensity  $-4$ )  $T$  and  $t$  increased about 1.1 log unit. The glow remained maximal during the illumination. During the ensuing dark period  $T$  and  $t$  decreased to the same values as before the illumination. After the eye had been kept in darkness for 16 min the glow diminished, indicating that the pigment migrated proximally, and  $T$  increased about 2.9 log units. When the eye was again exposed to the adapting light there was no change in  $T$  and  $t$ .

*Variations in transpigment threshold during pigment migration in the illuminated eye.* In some experiments the pigment movement during the illumination was associated with a decrease in  $T$ . In one example (Fig. 20)  $T$  decreased 0.7 log unit when the eye was exposed to the adapting light (log relative intensity  $-1$ ). During the first illumination period  $t$  decreased 2.0 log units. When the adapting light was turned off  $T$  reached a value that was 2.2 log units higher than before the illumination.

The maximal decrease in  $T$  when the pigment migrated during illumination was 0.7 log unit. No increase in  $T$  exceeding 0.1 log unit was found. The decrease in  $T$  which began simultaneously with the diminution of the glow was too large to be attributed to the inaccuracy of the threshold determinations. Furthermore,  $T$  did not decrease during the second illumination period when the pigment was already located proximally at the beginning of the illumination. These observations suggest that the decrease in  $T$  was related to the inward pigment movement. Experiments were done to test if the decrease in  $T$  was caused by the adapting light being attenuated more than the test light. Such a difference in attenuation could possibly have occurred since the optical path for the adapting light was not completely identical with that of the transpigment test light. The optical paths for the adapting and test lights were reversed for about one minute before and after the decrease in  $T$ . It was then found that the values for  $T$  were almost the same irrespective of which of the two optical paths

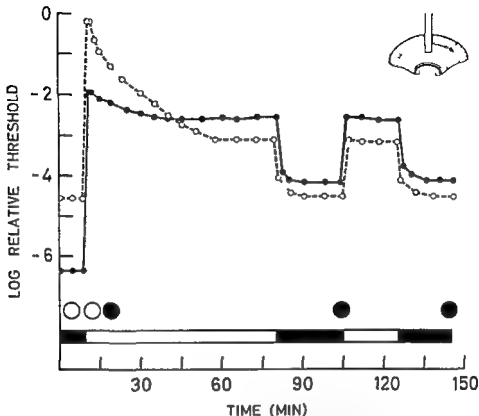


Fig 20  $T$  and  $t$  before during and after illumination (*Deilephila elpenor*)  $T$  decreased during first illumination period (For explanation of symbols see page 15)

was used for testing  $T$ . It is therefore unlikely that the decrease in  $T$  was caused by an unequal attenuation of the test and adapting lights.

In most of the experiments in which  $T$  decreased during the illumination as in the experiment shown in Fig 20 the relative change in the infrapigment threshold during the illumination exceeded the difference in the transpigment threshold before and after the illumination. This result indicates that in some eyes the increase in receptor sensitivity overcompensates for the increase in light attenuation by the pigment. Such an overcompensation may be one reason why in some experiments  $T$  decreased during the illumination.

*Neutral density filters substituting for pigment screening effect* In a series of experiments the relation between threshold change and change in adapting light intensity was determined. This increment threshold function was obtained by determining  $T$  and  $t$  at various adapting light intensities. The experiments were made on intact eyes in which the pigment remained distally as indicated



by a maximal glow, and also on eyes from which the distal pigments and cornea had been removed. There was thus no change in light screening by the pigment during the experiments. In both *Celerio* and *Deilephila* there was an approximately linear relationship between the change in log relative adapting light intensity and the change in log  $T$  and  $t$  from log relative adapting light intensity  $-1$  to about  $-4$ . In experiments on 9 intact eyes from *Deilephila* and on 11 eyes of the same species from which the distal pigments and the cornea had been removed a change in log relative adapting light intensity from  $-1$  to  $-4$ , i.e. 3.0 log units, caused a change in  $T$  and  $t$  ranging from 3.0 to 3.6 log units. The average change in both the intact eyes and those operated on was 3.2 log units. Thus a 1.0 log unit change in the adapting light intensity caused a change in  $T$  and  $t$  of 1.0 to 1.2 log unit (mean 1.07 log unit). This result supports the assumption that the increase in receptor sensitivity compensates and in some eyes possibly contributes to a slight overcompensation for the increase in light screening when the pigment migrates proximally.

Figure 21 shows an experiment on an eye from which the distal pigments were removed together with the cornea. A filter substituted for the pigment in that it attenuated the adapting light and the transpigment test light but not the infrapigment test light. At the beginning of the experiment no filter was used. The eye was first kept in darkness, and was then exposed to the adapting light (log relative intensity  $-1$ ). The increase in  $T$  and  $t$  was completed within the first minute of the illumination after which  $T$  and  $t$  were almost constant. When the eye had been exposed to the adapting light for about 5 min a filter attenuating the light by 3 log units was placed in front of the eye. Upon insertion of the filter  $t$  decreased about 3 log units while  $T$  first increased slightly and then gradually decreased to about the same value as before the insertion of the filter. The adaptation of the receptors to the lower light intensity was not completed until after 10 to 15 min. During the early part of this period the receptor sensitivity had not increased sufficiently to compensate for the light attenuation by the filter which caused the initial increase in  $T$ . No similar increase in  $T$  was seen in the experiments on intact eyes (see Figs 12, 15, 16 and 20) presumably because the pigment migration in the intact eye was sufficiently slow for the increase in receptor sensitivity to compensate for the increase in light attenuation at any time during the experiment.

In the experiment shown in Fig. 21, when the light was turned off  $t$  decreased to the same value as that found before the illumination. The receptors thus dark adapted completely.  $T$  decreased to a value that was about 3 log units higher than before the light adaptation since the test light was attenuated by the filter. When the filter was removed  $T$  decreased about 3 log units. When the eye was exposed a second time to the adapting light and then again kept in dark

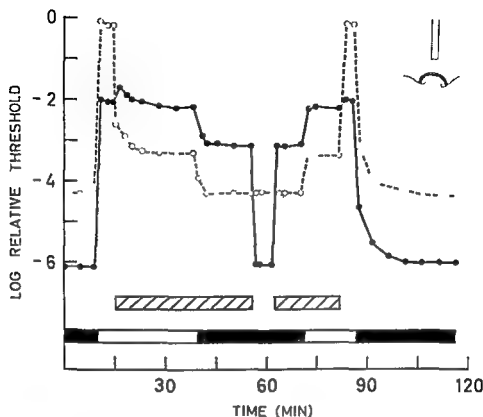


Fig 21  $T$  and  $t$  before during and after illumination of eye from which distal pigments had been removed (*Deilephila elpenor*) Neutral density filter substituted for pigment migration (For explanation of symbols see page 15)

ness  $T$  and  $t$  reached approximately the same values as those found earlier in the experiment. The experiment showed that the threshold changes observed during the pigment movements in intact eyes could be approximately reproduced when filters were substituted for the variations in light screening by the pigment. It should be noted however that the increase in light attenuation caused by the pigment migration may not be identical to that caused by neutral density filters (page 29). Therefore such filters probably do not completely reproduce the changes in the stimulating light caused by the pigment movements.

## DISCUSSION

Little is known about the relation between pigment migration and receptor sensitivity during light adaptation. Results of investigations on frogs (Dodt

and Jessen 1961 Back Donner and Reuter 1965) suggest that the pigment migration from the dark adapted to the light adapted position is associated with an increase in the sensitivity of the rods (see also Donner and Reuter 1965). The relation between the screening effect and the receptor sensitivity during light adaptation in the compound eye was recently studied by Post and Goldsmith (1965), but except for a brief previous report (Höglund 1963 b) no direct observations of the changes in light attenuation and their effect on the receptor sensitivity seem to have been reported.

The present experiments show that the screening effect of the pigment, and the receptor sensitivity, increase when the pigment migrates in the proximal direction during the adapting illumination. The observations strongly suggest that the increase in receptor sensitivity compensates for the increase in light screening so that the transpigment threshold changes little during the pigment movement. The same conclusion was also reached by Post and Goldsmith (1965) from their experiments on *Galleria*.

By the technique used in the present study variations in the receptor light sensitivity could be directly determined during pigment movements. In the assessment of results obtained by infrapigment light stimulation it should be considered that the intensity of the light illuminating retinulae close to the fiber tip is higher than the intensity of the light illuminating retinulae further away from the fiber. The angle of incidence on the rhabdomeres also varies with the distance from the fiber tip. Furthermore the intensity, angle of incidence on the rhabdomeres and the spectral energy distribution of the infrapigment light stimulus may be different from corresponding parameters of the transpigment light stimulus. These differences probably did not significantly influence the threshold measurements because control experiments (page 44) showed that a change in adapting light intensity caused about the same relative change in the infrapigment threshold as in the transpigment threshold. It is possible however that in some experiments depending on the position of the fiber tip the group of receptors that gave rise to the main part of the response to light from the fiber may not have been identical with the group of receptors that gave rise to the transpigment threshold response. If in such an experiment the pigment migrated more in some ommatidia than others the observed change in transpigment threshold may not have been a correct measure of the change in light screening that caused the observed change in the infrapigment threshold. Furthermore the pigment migration may cause changes in the light transmitted through the pigment that do not affect the infrapigment test light (page 29). For these reasons no definite conclusions have been drawn from a comparison between the numerical values for the relative changes in the infrapigment and transpigment thresholds.

The assumption that in some eyes the increase in receptor sensitivity overcompensates for the increase in screening effect by the pigment (page 43) is partially based on a comparison between the numerical values for the relative changes in the infrapigment and transpigment thresholds. Even though support for this assumption was obtained from measurements of the infrapigment and transpigment thresholds at various adapting light intensities (page 44) the experimental results are only regarded to indicate that such an overcompensation sometimes occurs. Similar findings were obtained in *Galleria* by Post and Goldsmith (1965). They found that in some eyes the inward pigment migration during illumination was accompanied by an increase in (transpigment) threshold and that a change of one log unit in adapting light intensity caused a smaller change in threshold. In the present study the change in log relative threshold sometimes exceeded the change in log relative adapting light intensity and the transpigment threshold decreased during the movement.

The report that an electrical potential change can be recorded when the isolated corneal pigment cell preparation from *Erebus* is exposed to light (Svaetichin, Fernandez-Moran and Jonasson 1956) raises the question whether in the present study part of the decrease in infrapigment threshold was caused by a decrease in the light intensity necessary to elicit a response from the pigment cells. It is unlikely, however, that more than a small part of the decrease in the infrapigment threshold reported here was caused by such a change in pigment cell threshold, as evidenced by the results of experiments made on eyes from which the cornea and a large part of the pigment cells were removed (page 44). In these eyes, as in intact eyes, a change in light attenuation of one log unit caused a threshold change of one or slightly more than one log unit, which suggests that the greater part of the decrease in the infrapigment threshold in intact eyes was caused by the increase in receptor sensitivity. It cannot be excluded, however, that a decrease in threshold of an electrical response from the pigment cells in intact eyes contributed to the threshold decrease by a few tenths of a log unit.

Schneider (1964) in his experiments on *Carauatus* measured the amplitude of the response to (transpigment) test flashes of constant intensity. He suggested that the slow decrease in amplitude observed during illumination of a previously dark adapted eye may be due to positional changes of the screening pigment. It is possible that this slow decrease corresponds to the slow changes in threshold sometimes found during the inward pigment movement when moth eyes are exposed to light. In locusts the initial increase in (transpigment) threshold when the eye is exposed to an adapting light is followed by a slow decrease (Burr, Catton and Cosens 1964). The threshold changes in the locust eye were determined by measuring the test light intensity needed to evoke a spike dis-

change in the nervous system Burt, Catton and Cosens (1964) found that the time course of these changes resembles the time course of a DC shift recorded in the reticular cell zone (Burt and Catton 1964). These variations in DC potential are abolished after removal of the optic lobe (Burt and Catton 1964) which indicates that the slow threshold decrease is not caused by pigment movements.

The experiments described in the present paper have investigated the relation between the receptor sensitivity and the screening effect by the pigment. It should be noted that the receptor sensitivity may also be affected by other factors such as the activity of the optic lobe and perhaps also by endocrine organs. In the water beetle *Dytiscus* the threshold of the dark adapted eye to (transpigment) light stimuli is about a thousand times higher during the day than during the night and the difference in sensitivity is unrelated to variations in pigment position (Jahn and Wulff 1941, 1943). Furthermore, activity in the optic lobe has been reported to influence the electrical response to light stimulation in several other insect species e.g. in flies (Autrum 1958, Burt and Catton 1964) in locusts (Burt and Catton 1964), and in the butterfly *Heliconius* (Swihart 1964).

Kuiper (1962) has suggested that the pigment protects the receptor cells from overillumination. The observation that the receptor sensitivity increases during the inward pigment migration, and thereby compensates for the decreasing intensity of the light reaching the rhabdomeres suggests in accordance with Kuiper's hypothesis, that one function of the migrating pigment is to reduce the intensity of the receptor stimulation with little loss in the sensitivity to (transpigment) light stimuli.

In moths the changes in sensitivity to (transpigment) light stimuli are usually completed rapidly after a sudden change in the adapting light intensity, e.g. when a shadow is cast on the eye even though the pigment migration is slow. However the change in sensitivity is rapidly completed only if the change in adapting light intensity is moderate. If a previously brightly illuminated eye with the pigment in the proximal position is suddenly exposed to a very low adapting light intensity the pigment remains for some time in the proximal position. Until the pigment has migrated to a more distal position the light may be attenuated to such an extent that no response is evoked in the reticular cells even if they have gained the same sensitivity as in a completely dark adapted eye. The intensity of the light reaching the rhabdomeres may also be too low to excite the reticular cells during pigment movements that are not induced by changes in the adapting light intensity such as rhythmic pigment movements. When the change in the adapting light intensity is slow, e.g. during sunrise and sunset the pigment movements follow the change in the light intensity so that

the intensity of the light reaching the rhabdomeres changes less than the adapting light intensity

It is of interest that in the frog the screening effect by the pigment and thereby the rhodopsin content in the illuminated eye varies with the pigment position (Bick, Donner and Reuter 1967). This finding together with the report that the pigment position affects the threshold of the illuminated eye (Dodt and Jessen 1961) suggests that the relation between pigment light screening and receptor sensitivity may be partially similar in arthropod and vertebrate eyes

## SUMMARY

1 The relation between the photoreceptor light sensitivity and the positional changes of the retinal screening pigment was studied in the compound eye of the moths *Celerio euphorbiae* and *Deilephila elpenor* (Sphingidae)

2 Relative thresholds were measured electrophysiologically in darkness and during illumination by *a* test light passing through the cornea and distal pigments (the transpigment threshold) and *b* test light conducted through a glass fiber, the tip of which was placed below the accessory pigment (the infrapigment threshold)

3 The pigment position in histological sections of eyes exhibiting maximal or minimal glow was determined. It was found that the proximal and distal borders of the accessory pigment were located more proximally in eyes with minimal glow than in eyes with maximal glow. Changes in glow size between maximal and minimal were therefore used as index of pigment movements. No movements of pigment near the basement membrane exceeding a few  $\mu\text{m}$  were found.

4 The threshold measurements showed that in dark adapted eyes the infrapigment and transpigment thresholds were constant as long as the pigment remained in the extreme distal position. An inward pigment migration in dark adapted eyes kept in darkness was associated with an increase in the transpigment threshold while the infrapigment threshold remained constant. An outward pigment migration was associated with a decrease in the transpigment threshold.

5 The infrapigment and transpigment thresholds reached a steady value in less than one minute after the onset of adapting light. The thresholds then remained almost constant as long as the pigment remained in the extreme distal position. Cessation of the adapting light with the pigment in the extreme distal position was followed by a gradual decrease in the infrapigment and the transpigment thresholds to the same values as those found before the illumination. Almost the same time course of threshold change was found in eyes from which the distal pigments had been removed.

6 The inward pigment migration during exposure to adapting light was associated with a decrease in infrapigment threshold. The transpigment threshold remained constant or decreased slightly during the pigment migration. Upon

cessation of the adapting light the infrapigment threshold decreased to the same value as that found before the illumination although the pigment did not migrate distally during the dark period. The transpigment threshold decreased to a value that was higher than before the illumination.

Upon re-exposure of the eye to the adapting light the infrapigment threshold increased to about the same value as that observed at the end of the preceding illumination, provided the pigment did not migrate during the intervening dark period. When there was an inward pigment migration during the intervening dark period the infrapigment threshold upon re-illumination increased to a value lower than at the end of the preceding illumination. The transpigment threshold increased to about the same value as that observed at the end of the preceding illumination irrespective whether the pigment migrated during the intervening dark period. However, if the infrapigment threshold remained the same in the illuminated as in the dark adapted eye the value of the transpigment threshold upon re-illumination was higher than the value observed at the end of the preceding illumination.

7 In most experiments in which the transpigment threshold decreased during the pigment migration occurring during exposure to adapting light the relative change in infrapigment threshold during the pigment movement exceeded the difference between the transpigment threshold before and after illumination. Increment threshold experiments showed that a change in the adapting light intensity of 1.0 log unit caused the infrapigment and transpigment thresholds to change 1.0 to 1.2 log unit. The threshold changes observed during the pigment migration could be approximately reproduced when neutral density filters were substituted for the variations in light screening by the pigment.

8 The experimental results show that in dark adapted eyes kept in darkness pigment movements may occur with no change in the receptor light sensitivity. The light attenuation varies with the pigment position and thus causes variations in the sensitivity to (transpigment) light stimuli.

During illumination the pigment migrates proximally. Due to the increase in light attenuation the intensity of the light reaching the rhabdomeres gradually decreases. The photoreceptors adapt to the decreasing stimulus intensity with an increase in their sensitivity. The sensitivity to (transpigment) light stimuli changes only slightly during the pigment movement because the increase in receptor sensitivity usually compensates for the increase in light attenuation. The increase in receptor sensitivity may in some eyes contribute to an overcompensation for the increase in light attenuation. The increase in light attenuation is not compensated for if the inward pigment migration continues after the receptors have attained the same sensitivity as in the dark adapted eye.



The research reported in this document has been sponsored by the Air Force Office of Scientific Research under Grant AF EOAR 65—9 through the European Office of Aerospace Research (OAR) United States Air Force by the Swedish Medical Research Council by Reservationsanslaget Karolinska Institutet and by Svenska Sällskapet för Medicinsk Forskning

## REFERENCES

- AUTRUM H Die spektrale Empfindlichkeit der Augenmutation white apricot von *Calliphora erythrocephala* *Biol Zbl* 1955 74 515—521
- AUTRUM H Electrophysiological analysis of the visual systems in insects *Exp Cell Res* 1958 Suppl 5 426—439
- AUTRUM H I AUTRUM and C HOFFMANN Komponenten im Retinogramm von *Calliphora* und ihre Abhängigkeit von der Spektralfarbe *Biol Zbl* 1961 80 513—547
- AUTRUM H and D BURKHARDT Spectral sensitivity of single visual cells *Nature Lond* 1961 190 639
- AUTRUM H and U GALLWITZ Zur Analyse der Belichtungspotentiale des Insektenauges *Z vergl Physiol* 1951 33 407—435
- AUTRUM H and C HOFFMANN Diphasic and monophasic responses in the compound eye of *Calliphora* *J Insect Physiol* 1960 4 122—127
- BACK I K O DONNER and T REUTER The screening effect of the pigment epithelium on the retinal rods in the frog *Vision Res* 1965 5 101—111
- BALDWIN W F and J B SUTHERLAND Extreme sensitivity to low level X rays in the eye of the cockroach *Blaberus* *Radiat Res* 1965 74 513—518
- BENNET R and A D MERRICK Migration of the proximal retinal pigment in the crayfish in relation to oxygen deficiency *Biol Bull mar biol Lab Woods Hole* 1937 67 168—177
- BERNHARD C G Isolation of retinal and optic ganglion response in the eye of *Dytiscus* *J Neurophysiol* 1942 5 37—48
- BERNHARD C G On the functional significance of the pigment migration in the compound eye *Acta physiol scand* 1963 59 Suppl 213 29
- BERNHARD C G G HOGLUND and D OTTOSON On the relation between pigment position and light sensitivity of the compound eye in different nocturnal insects *J Insect Physiol* 1963 9 573—586
- BERNHARD C G and D OTTOSON Comparative studies on dark adaptation in the compound eyes of nocturnal and diurnal Lepidoptera *J gen Physiol* 1960 a 44 195—203
- BERNHARD C G and D OTTOSON Studies on the relation between the pigment migration and the sensitivity changes during dark adaptation in diurnal and nocturnal Lepidoptera *J gen Physiol* 1960 b 44 205—215
- BERNHARD C G and D OTTOSON Quantitative studies on pigment migration and light sensitivity in the compound eye at different light intensities *J gen Physiol* 1964 47 465—478
- BRUN G H P de and D J CRISP The influence of pigment migration on vision of higher Crustacea *J exp Biol* 1957 34 447—463
- BIGNON E and N POROFF Les yeux des insectes nocturne *Archs Anat microsc Morph exp* 1914 16 261—304
- BULLOCK T H and G A HORRIDGE *Structure and function in the nervous systems of invertebrates* San Francisco and London Freeman 1965 7
- BURKHARDT D Spectral sensitivity and other response characteristics of single visual cells in the arthropod eye *Symp Soc exp Biol* 1962 16 86—109
- BURKHARDT D Colour discrimination in insects In *Advances in insect physiology* Editors BEAMENT J W L J E TREHERNE and V B WIGGLESWORTH London and New York Academic Press 1964 2 131—173

The research reported in this document has been sponsored by the Air Force Office of Scientific Research under Grant AF EOAR 65—9 through the European Office of Aerospace Research (OAR) United States Air Force by the Swedish Medical Research Council by Reservationsanslag t Karolinska Institutet and by Svenska Sällskapet for Medicinsk Forskning

# REFERENCES

- AUTRUM H Die spektrale Empfindlichkeit der Augenmutation white apricot von *Calliphora erythrocephala* *Biol Zbl* 1955 74 515—524
- AUTRUM H Electrophysiological analysis of the visual systems in insects *Exp Cell Res* 1958 Suppl 5 426—439
- AUTRUM H I AUTRUM and C HOFFMANN Komponenten im Retinogramm von *Calliphora* und ihre Abhängigkeit von der Spektralfarbe *Biol Zbl* 1961 80 513—547
- AUTRUM H and D BURKHARDT Spectral sensitivity of single visual cells *Nature Lond* 1961 190 639
- AUTRUM H and U GALLWITZ Zur Analyse der Lichtungspotentiale des Insektenauges *Z vergl Physiol* 1951 33 407—435
- AUTRUM H and C HOFFMANN Diphasic and monophasic responses in the compound eye of *Calliphora* *J Insect Physiol* 1960 4 192—197
- BACK I K O DONNER and T REUTER The screening effect of the pigment epithelium on the retinal rods in the frog *Vision Res* 1965 5 101—111
- BALDWIN W F and J B SUTHERLAND Extreme sensitivity to low level X rays in the eye of the cockroach *Blaberus* *Radiat Res* 1965 34 513—518
- BENNET R and A D MERRICK Migration of the proximal retinal pigment in the crayfish in relation to oxygen deficiency *Biol Pulmar biol Lab Woods Hole* 1937 67 168—177
- BERNHARD C G Isolation of retinal and optical lion response in the eye of *Dytiscus* *J Neurophysiol* 1942 5 37—48
- BERNHARD C G On the functional significance of the pigment migration in the compound eye *Acta physiol scand* 1963 59 5 213—227
- BERNHARD C G G HOOGLUND and D OTT On the relation between pigment position and light sensitivity of the compound eye of nocturnal insects *J Insect Physiol* 1963 9 573—586
- BERNHARD C G and D OTTOSOV Comparative studies on dark adaptation in the compound eyes of nocturnal and diurnal *Lepidoptera* *J gen Physiol* 1960 a 44 195—203
- BERNHARD C G and D OTTOSOV Studies on the relation between the pigment migration and the sensitivity changes during dark adaptation in diurnal and nocturnal *Lepidoptera* *J gen Physiol* 1960 b 44 205—215
- BERNHARD C G and D OTTOSOV Quantitative studies on pigment migration and light sensitivity in the compound eye at different light intensities *J gen Physiol* 1964 47 465—478
- BRUCE G H P de and D J CRISP The influence of pigment migration on vision of higher Crustacea *J exp Biol* 1957 31 447—463
- BURGTON E and N POPOFF Les yeux des insectes nocturnes *Arch Anat microsc Morph exp* 1914 16 261—304
- BILLOCK T H and G A HORRIDGE *Structure and function in the nervous systems of invertebrates* San Francisco and London Freeman 1965 2
- BURKHARDT D Spectral sensitivity and other response characteristics of single visual cells in the arthropod eye *Symp Soc exp Biol* 1967 16 86—109
- BURKHARDT D Colour discrimination in insects *In Advances in insect physiology* Editors BEAMENT J W L J E TREHNER and V B WIGGLESWORTH London and New York Academic Press 1964 2 131—173

- BURTT E T and W T CATTON Potential changes in the eye and optic lobe of certain insects during light and dark adaptation *J Insect Physiol* 1964 10 865—886
- BURTT, E T W T CATTON and D J COSENS Correlation between electrical potential and visual threshold changes in an insect eye *J Physiol Lond* 1964 170 57 P—58 P
- BYZOV A L O YU ORLOV and I A UTINA (Study of eye adaption in cephalopod molluscs in Russian) *Biophysika* 1963 7 318—327 English abstract in *Biophysics* 1963 8 187
- COLLINS D L Iris pigment migration and its relation to behavior in the codling moth *J exp Zool* 1934 69 165—197
- DAY M F Pigment migration in the eyes of the moth *Ephestia kuehniella* Zeller *Biol Bull mar biol Lab Woods Hole* 1941 80 275—291
- DEMOLL R Über die Wanderung des Irispigments im Facettenauge *Zool Jb Abt allg Zool Physiol* 1911 30 169—180
- DEMOLL R *Die Sinnesorgane der Arthropoden ihr Bau und ihre Funktion* Braunschweig Vieweg 1917
- DIXON W J and F J MASSEY Jr *Introduction to statistical analysis* New York Toronto and London Mc Graw Hill 1957
- DODT E and K H JESSEN Das adaptive Verhalten der Froschnetzhaut untersucht mit der Methode der konstanten elektrischen Antwort *Vision Res* 1961 1 228—243
- DOYNER K O and T REUTER The dark adaptation of single units in the frog's retina and its relation to the regeneration of rhodopsin *Vision Res* 1965 5 615—632
- DUKE ELDER S The eye in evolution In *System of ophthalmology* Editor DUKE ELDER S London Kimpton 1958 I
- EDWARDS D K Light dark adaptation and glow in abnormal moth eyes without pigment *Nature Lond* 1964 202 621—622
- EPHRASSI B and G W BEADLE A technique of transplantation for *Drosophila* *Am Nat* 1936 70 218—225
- EYNER S *Die Physiologie der facettierten Augen von Krabben und Insecten* Leipzig and Vienna Deuticke 1891
- FRISCH K von Studien über die Pigmentverschiebung im Facettenauge *Biol Zbl* 1908 28 667—671
- GATENBY J H and H W BEANS *The microtommist's vade mecum* London Churchill 1950
- GOLDSMITH T H The nature of the retinal action potential and the spectral sensitivities of ultraviolet and green receptor systems of the compound eye of the worker honeybee *J gen Physiol* 1960 43 775—799
- GOLDSMITH T H The course of light and dark adaptation in the compound eye of the honey bee *Comp Biochem Physiol* 1963 10 227—237
- GOLDSMITH T H The visual system of insects In *The physiology of Insecta* Editor ROCKSTEIN M London and New York Academic Press 1964 1 397—462
- GOLDSMITH T H Do flies have a red receptor? *J gen Physiol* 1965 49 265—287
- HARTLINE H K H G WAGNER and E F Mac NICHOL Jr The peripheral origin of nervous activity in the visual system *Cold Spring Harb Symp quant Biol* 1952 17 125—141
- HESLOP I R P Revised indexed check list of the British Lepidoptera *Entomologist's Ga* 1959 10 177—187
- HESLOP I R P Revised indexed check list of the British Lepidoptera *Entomologist's Ga* 1960 11 55—66
- HOFFMAN C Belichtungspotentiale der Insekten und Sauerstoffdruck *Verh dt zool Ges* 1960 Suppl 23 220—225
- HOFFMANN C and H LANGER Die spektrale Augenempfindlichkeit der Mutante chalky von *Calliphora erythrocephala* *Naturwissenschaften* 1961 48 605

- HÖGLUND G. Glow sensitivity changes and pigment migration in the compound eye of nocturnal Lepidoptera *Life Sciences* 1963 a 2 275—280
- HÖGLUND G. Receptor sensitivity and pigment position in the compound eye of nocturnal Lepidoptera *Life Sciences* 1963 b 2 862—865
- HÖGLUND G. Pigment migration and retinal sensitivity. In *Functional Organization of the Compound Eye* Editor BERNHARD C G. *Wenner Gren Center int Symp Ser* London Pergamon Press 1966 7 77—88
- HORSTMANN E. Die tagesperiodischen Pigmentwanderungen im Facettenauge von Nachschmetterlingen *Biol Zbl* 1935 55 93—97
- JAHN T L and V J WULF. Retinal pigment distribution in relation to a diurnal rhythm in the compound eye of *Dytiscus* *Proc Soc exp Biol Med* 1941 48 656—660
- JAHN T L and V J WULF. Allocation of electrical responses from the compound eye of grasshoppers *J gen Physiol* 1942 26 75—88
- JAHN T L and V J WULF. Electrical aspects of a diurnal rhythm in the eye of *Dytiscus fasciventris* *Physiol Zool* 1943 16 101—109
- KIESEL A. Untersuchungen zur Physiologie des facettierten Auges *Sber Akad Wiss Wien math naturh Kl* 1894 103 97—139
- KLINGEBEL K H. Über die Lichtreaktionen von Augenmutation rassen der Mehlmotte *Ephestia kuehniella* Zeller *Biol Zbl* 1938 58 631—646
- KLIPPER J W. The optics of the compound eye *Symp Soc exp Biol* 1962 16 58—71
- LANGER H. Untersuchungen über die Grösse des Stoffwechsels isolierter Augen von *Calliphora erythrocephala* Meigen *Biol Zbl* 1967 81 691—720
- LANGER H and C HORRMANN. Elektro und stoffwechselphysiologische Untersuchungen über den Einfluss von Ommochromen und Pteridinen auf die Funktion des Facettenauges von *Calliphora erythrocephala* *J Insect Physiol* 1966 12 357—387
- LEYDIG F. *Das Auge der Gliedertiere* Tübingen Laupp 1864
- LUDTKE H. Dunkeladaptation und Verschiebung der Helligkeitswerte im Auge von *Notopecta glauca* L. *Z Naturf* 1954 9 B 159—163
- MERKER E. Die Pigmentverschiebungen im Netzauge der Insekten unter dem Einfluss von ultraviolettem Licht *Zool Jb Abt allg Zool Physiol* 1929 46 297—374
- PARKER G H. The movements of the retinal pigment *Ergebn Biol* 1937 9 239—291
- PO T C T Jr and T H GOLDSWORTHY. Pigment migration and light adaptation in the eye of the moth *Galleria mellonella* *Biol Bull mar biol Lab Woods Hole* 1965 128 473—487
- ROMEIS B. *Mikroskopische Technik* Munich Leibniz 1948
- RLCA P. Photoreceptor cell response and flicker fusion frequency in the compound eye of the fly *Lucilia sericata* (Meigen) *Biol Bull mar biol Lab Woods Hole* 1961 120 375—383
- SCHNEIDER G. Untersuchungen zur Analyse der retinalen Flimmerpotentiale von *Caeranus morosus* *Z vergl Physiol* 1964 49 195—269
- SMITH J C and D J KIMMELDORF. The bioelectrical response of the insect eye to beta radiation *J Insect Physiol* 1964 10 839—847
- SNODGRASS R E. *Principles of insect morphology* New York and London Mc Graw Hill 1935
- STEPANOWSKA M. La disposition histologique du pigment dans les yeux des Arthropodes sous l'influence de la lumière directe et de l'obscurité complète *Recl ool suisse* 1890 5 151—200
- SVÄTICHIN G H FERNANDEZ MORAN and R JOHASSOV. Electrophysiological and electron microscope studies of the insect compound eye *20th Int physiol Congr* 1956 2 866—867
- SWINART S L. The nature of the electroretinogram of a tropical butterfly *J Insect Physiol* 1964 10 547—562

- TELLALA O Histologische und physiologische Untersuchungen über die photomechanischen Erscheinungen in den Augen der Lepidopteren *Suomal Tiedekat Toim* (*Annls Acad scient fennicae*) A 4 1954 24 1—69
- YAGI N and N Koyama *The compound eye of Lepidoptera* Tokyo Shunkyo Press 1963







**ACTA PHYSIOLOGICA SCANDINAVICA**

**VOL. 69 SUPPLEMENTUM 233**

**ESTERASES OF THE RAT  
ADENOHYPHYSIS**

**CELLULAR LOCALIZATION AND ACTIVITY IN RELATION  
TO SECRETORY FUNCTIONS**

**BY**

**TAPANI VANHA PERTTULA**

**TURKU 1966**



ACTA PHYSIOLOGICA SCANDINAVICA

VOL 69 SUPPLEMENTUM 283

FROM THE DEPARTMENT OF ANATOMY UNIVERSITY OF TURKU  
TURKU FINLAND

# ESTERASES OF THE RAT ADENOHYPOPHYSIS

CYTOPLASMIC LOCALIZATION AND ACTIVITY IN RELATION  
TO SECRETORY FUNCTIONS

BY

TAI ANI VANHAPFERTTULA

TURKU 1966

Turku  
Kirjapaino Polytypos  
1966

## PREFACE

The present study was carried out at the Department of Anatomy, University of Turku in 1963—1966

I wish to express my sincere thanks to Assistant Professor Vaino K Hopsu M D, who suggested me this field of study and who gave me encouragement and valuable advice throughout the investigation. I am also grateful to Professor Mikko Nieminen, M D, the present Head of the Department for his critical and valuable suggestions in the preparation of the manuscript.

My best thanks are due to my colleagues at the Department for pleasant collaboration and friendly dealings. I wish warmly to thank the technical personnel of the Department especially Mrs Marja Ovasla for her skilful technical assistance and Mr Paavo Viljanen for the great trouble he took with the photographs.

I am further indebted to Dr William Waddell M D from the Department of Pharmacology, University of North Carolina, Chapel Hill, USA for checking the language of the manuscript and his interest in my work. I am grateful to Mrs Aili Rytönen, M A and her assistants at the Library of the Medical Faculty for their help in obtaining literature. My thanks are also due to Mr Heikki Loppinen M A for his advice in statistical problems and to Mr Unto Mäkiö M Sc for the generous supply of the steroid hormones of N V Organon used in the present work.

This investigation was financially supported by a grant from the Jalmar and Rauha Ahokas Foundation which is gratefully acknowledged.  
Turku September 1966

*Tapani Vauhkonen*



# CONTENTS

	Page
INTRODUCTION	7
HISTOLOGY OF THE RAT ADENOHYPOPHYSIS	8
<i>Literature review</i>	8
Parenchymal cells	9
Cleft epithelial cells	12
Vascular and perivascular cells	13
<i>Own investigations</i>	13
Material and methods	13
Results	14
Characteristics of the parenchymal cell types	14
Other cell constituents of the adenohypophysis	19
Discussion	20
HISTOCHEMISTRY OF CARBOXYLIC ESTERASES IN THE ADENOHYPOPHYSIS	21
<i>Literature review</i>	22
Classification and characterization of carboxylic esterases	22
Adenohypophyseal carboxylic esterases	23
Cholinesterases	23
Non specific esterases	23
Changes in experimental conditions	25
<i>Own investigations</i>	26
Material	26
Histochemical staining methods	26
Modifiers for the classification of esterases	27
Results	27
Carboxylic esterases in normal rat pituitary sections	27
Hydrolysis of thiocholine esters	30
Hydrolysis of naphthol ester · thioacetic acid and 5 bromoindoxyl acetate	31
Changes in experimental conditions	42
Cholinesterases	42
E600 sensitive non specific esterases	43
E600 resistant non specific esterases	46
Discussion	49
Cholinesterases	48
E600 sensitive non specific esterases	48
E600 resistant non specific esterases	51



FRACTIONATION OF CARBOXYLIC ESTERASES IN THE RAT ADENO HYPOPHYSIS	53
<i>Literature review</i>	53
Earlier fractionation studies of esterases	53
<i>Own investigations</i>	54
Material and methods	54
Results	56
Studies with tissue homogenate	56
Starch gel electrophoresis	59
Histochemical staining of the starch gel slabs	59
Studies with starch gel eluates	63
Correlation of esterase reactions and zymograms	64
DEAE cellulose column chromatography	67
Discussion	68
QUANTITATIVE ESTIMATION OF CARBOXYLIC ESTERASES IN EXPERI MENTAL CONDITIONS	73
<i>Own investigations</i>	73
Material and methods	73
Statistical treatment of the results	75
Results and discussion	78
GENERAL DISCUSSION	82
SUMMARY	97
REFERENCES	91

## INTRODUCTION

Several different types of parenchymal cells can be demonstrated in the rat anterior pituitary gland by a number of histological and histochemical techniques. However these techniques do not specifically demonstrate the pituitary hormones themselves. Therefore an experimental approach has frequently been used in order to localize the site of synthesis and/or storage of a specific hormone to a certain cell type. This usually includes observations on the pituitary cytology after total extirpation of different endocrine glands or after experimentally induced alterations in their functional status. During recent years a more direct approach has become available for the cellular localization of some pituitary hormones and this is based on the use of fluorescent labelled antibodies to these hormones.

Any synthesis and release of hormones must obviously rely on a series of reactions coupled with a number of more or less specifically acting enzyme systems. Unfortunately only a limited number of such enzymatic activities can be localized histochemically to a specific cell. Thus most of the enzymes active in protein and polysaccharide synthesis and consequently connected with the synthesis of the anterior pituitary hormones cannot be demonstrated at the cellular level. On the other hand a number of histochemically demonstrable enzyme activities have been found specifically distributed among the pituitary cells. The enzymes with an esterolytic capacity are especially active in the anterior pituitary gland but the role of this class of enzymes for hormone production has not been worked out. Only a few published reports deal with changes in histochemically demonstrable esterase activity in experimentally altered pituitary function. Quantitative measurements of the total esterase activity of the pituitary gland under experimental conditions are completely lacking.

Observations leading to the discovery of specific enzymes taking part in the hormone production or release may be expected to aid in the distinction of the pituitary cell types. However pure biochemical characterization of a specific enzyme of the pituitary gland is not suitable for

correlation of enzymic activity to the production of a certain hormone because of the multiple cellular composition of the gland. Microscopic histochemical investigation should be therefore combined with biochemical assays to explore the specific metabolic characteristics of the pituitary cells.

Histochemically demonstrable esterases give an example of a heterogeneous group of enzymes, whose biochemical characteristics can not be adequately described using conventional histochemical investigation. Biochemical separation of a tissue homogenate and further characterization of the partially purified esterase fractions using different substrates and modifier agents are necessary to complete an investigation of the esterases in a tissue like the pituitary gland. Quantitative determination of the esterase activity in different functional states of an endocrine gland is obviously of importance for an evaluation of the significance of the enzyme to hormone production.

# HISTOLOGY OF THE RAT ADENOHYPOPHYSIS

## LITERATURE REVIEW

### Parenchymal cells

The anterior pituitary gland secretes the following *protein hormones*: somatotrophic or growth hormone (STH), prolactin or lactotropic hormone (LTH), corticotrophic hormone (ACTH) and melanotropic or melanocyte stimulating hormone (MSH) in man (Morris et al 1956 Parres 1961 Hermanus et al 1964) and in species which have no intermediate lobe (Tixier Vidal 1963). The *glycoprotein hormones* produced by the pituitary gland are: follicle stimulating hormone (FSH), luteinizing hormone (LH) or interstitial cell stimulating hormone (ICSH) and thyrotrophic hormone (TSH). [The nomenclature follows the recommendation of the International Committee for the Nomenclature of the Adenohypophysis (van Oordt 1965)]

The localization of these hormones in the different cell types in the anterior pituitary gland has been an intriguing problem for many decades. From the abundant studies concerning the pituitary morphology in the rat only the most important contributions are presented schematically in Table I.

**Table I** Summary of the literature ascribing the production of different hormones to specific types in the rat pituitary gland. Included are the immunofluorescent studies in different species.

Hormone	Staining (Mallory)	Cell type (Herlant)	Special feature	Reference
STH	acidophil	$\alpha$	orangeophile carminophile phospholipid positive orangeophile (tetrachrome and Cleveland Wolfe staining) degranulation after thyroidectomy immunofluorescence (man)	Dawson 1954 Sanders & Rennels 1959 Herlant 1959, Rennels 1960 Pasteel & Herlant 1962  Knigge 1958  Leznoff et al 1960 Grumbach 1961 Pearl & van Noorden 1962 Rumke & Ladiges 1963
			immunofluorescence (ox, sheep rat, mouse)	

correlation of enzymic activity to the production of a certain hormone because of the multiple cellular composition of the gland. Microscopic histochemical investigation should be therefore combined with biochemical assays to explore the specific metabolic characteristics of the pituitary cells.

Histochemically demonstrable esterases give an example of a heterogeneous group of enzymes, whose biochemical characteristics can not be adequately described using conventional histochemical investigation. Biochemical separation of a tissue homogenate and further characterization of the partially purified esterase fractions using different substrates and modifier agents are necessary to complete an investigation of the esterases in a tissue like the pituitary gland. Quantitative determination of the esterase activity in different functional states of an endocrine gland is obviously of importance for an evaluation of the significance of the enzyme to hormone production.

hormone	Staining (Mallory)	Cell type (Herlant)	Special features	Reference
I or III	basophil	$\gamma$	PAS + central, round	Purves & Griesbach 1961, 1955 1956
			PAS red peripheral	Rennels 1967, 1963
			immunofluorescence (man)	Hildebrand et al 1967
				Midgley 1963
III	basophil	$\beta$		Koffler & Fogel 1964
				Pobyn et al 1964
			I AS + peripheral	Purves & Griesbach 1964 1965 1966
			PAS purple, central	Rennels 1967, 1963
			immunofluorescence (man)	Hildebrand et al 1967
			immunofluorescence (pig)	Koffler & Fogel 1964
				Della Corte & Biondi 1964

The site of the *somatotropic hormone* production in acidophilic cells of the pituitary gland was suggested first by the observation that acidophilic adenomas produce symptoms of acromegaly and gigantism (Cushing and Davidoff 1927). These findings have been specifically confirmed by immunofluorescent studies in man (Leznoff et al 1960 Grumbich 1963 Pearse and Van Noorden 1963b) and in different animals including the rat (Pumke and Ladiges 1965). In the rat hypophy is these cells rapidly lose their granules after thyroidectomy or after the administration of antithyroid drugs and the degranulation can be prevented by the administration of thyroxine (Zeckwer et al 1930 Purves and Griesbach 1946 Halmi 1960 Griesbach 1963). The acidophilic cell secreting *STH* is orangeophilic according to the classification of Dawson and Friedgood (1938).

Another type of acidophilic cell in a number of animals is carminophilic and erythrosinophilic. These cells are present also in the rat pituitary gland and are most active during pregnancy and lactation (Dawson 1963). The granules of these cells are depleted during the suckling of the litter which agrees with the earlier finding that suckling causes a rapid fall in the *lactotropic hormone* content of the pituitary gland (Grosvenor and Turner 1958). Based on the differential solubilities of the pituitary tropic hormones in trichloroacetic acid solutions Barnett et al (1961) have located *LTH* activity in the acidophilic series of rat pituitary cells. Immunologic confirmation for the localization of *LTH* in acidophils has also been performed in the rat pituitary gland (Emmatt et al 1963 Pumke and Ladiges 1965). According to Sanders and Rennels (1969) *LTH* is secreted by the orangeophilic variant of the acidophilic cells and *STH* by the carminophilic cells.

The studies concerning the cellular origin of the *corticotropic hormone* in different animal species have been recently summarized by Cirod (1964). All the cell types in the pituitary gland have been suggested at one time or another to be the site of *ACTH* secretion. Even the immunofluorescent investigations have given controversial results (Marshall 1961, Leznoff et al 1963 Pearse and Van Noorden 1963b). The cellular

site of ACTH production in the rat has been thought to reside in acidophilic cells (Kracht et al 1963a b). Autoradiographic studies by Siperstein (1963) and Knutson (1966) as well as electron microscopic studies by Farquhar (1957), Siperstein and Allison (1965) and Kurosumi and Kobayashi (1966), on the other hand indicate chromophobes as ACTH producers. A quite new site of the ACTH production in the rat was suggested by Herlant and his colleague. This was a chromophobe cell which contains a seed bed of erythrosomophilic granules at one end of the cell when stained by the tetrachrome method (Herlant 1960a). A definite hypertrophy of this cell type was found after adrenalectomy (Quenum and Herlant 1964) as well as after such drugs as amphenone and metopirone (Iacodot and Herlant 1960, Herlant and Klatersky 1963, Racadot 1963a c). These cells are involuted after cortisone treatment (Racadot 1963c). The histologic appearance of this cell type in the rat anterior pituitary gland greatly resembles that of LH cells in Herlant's tetrachrome method (Pasteels and Herlant 1962).

A different localization of gonadotropic hormones in the PAS positive cells was suggested by Purves and Griesbach (1954 1955). FSH seemed to be secreted by peripheral coarse granulated cells and LH by centrally situated larger, and fine-granulated cells. An opposite opinion of the location of LH and FSH activities in the rat pituitary gland has been presented however (Rennels 1955, 1963, Hildebrand et al 1957, Hellbrum et al 1961). By using a PAS methyl blue orange G method some cells are stained red and are situated peripherally in the so called 'ex zone'. An increase of this cell type after gonadectomy coincides with an increase in LH activity whereas high FSH activity can be found when PAS purple central gonadotrophs were increased in number. Strain differences may explain a part of this controversy (Rennels 1963).

Since the studies of Halmi (1950, 1952a b) and Purves and Griesbach (1954a, b, c, 1956) there has been a unanimous opinion of the site of the thyrotropic hormone production in the central basophilic cells, which have a specific affinity to aldehyde-fuchsin (Halmi 1950) and aldehyde-thionin (Eaget and Eccleston 1960, Ferrin and Murray 1963).

## Cleft epithelial cells

The anterior pituitary gland of the rat is separated from the intermediate lobe by a cleftlike space which is covered by epithelial cell linings on both sides. When the anterior lobe is separated mechanically from the intermediate and posterior lobes the anterior epithelium follows the anterior lobe tissue. Therefore some properties of these cell coverings must be presented. The lining cells of the cystlike cavities between anterior and posterior lobes in man secrete colloidal material into the cysts (Pomeroy 1940). The function of this colloid is still unknown. In the rat pituitary the sickle shaped cleft between anterior and intermediate lobes remains throughout the life of the animal.

The anterior and posterior epithelial linings have differences in their cellular characteristics (Ferrer 1956). The posterior epithelium lining the intermediate lobe varies from flattened cubical, or cylindrical to stratified epithelium and different cell types can be identified. Colloid droplets have been found in the apical zones of the cells and the colloid in the pituitary cleft is probably mainly the product of apocrine secretion of the cells. Increased secretion has been found after castration and ad

renalectomy The anterior epithelium = low Through fenestrations in the epithelial lining basophils, acidophils and capillaries can project into direct contact with the cleft remnants of the anterior pituitary cell transudation from the capillaries as well as mucus from the goblet cells have also been thought to contribute to the colloid formation (Ferrer 1956)

## Vascular and perivascular cells

The sinusoidal capillaries lead the blood from the base of the hypothalamus to the anterior pituitary gland The studies of Cappell (1929) have shown that the pituitary capillaries are formed by endothelial cells and, in the perisinusoidal space by perivascular cells which have the property of phagocytosis (Rinehart and Farquhar 1955 Farquhar 1961) Around these sinusoids fine reticular fibres can be seen in the homogeneous ground substance The fibres as well as the ground substance are PAS positive

The perisinusoidal spaces extend between the parenchymal cells thus allowing the cells a large surface area to liberate their granules The actual liberation of secretory granules of glandular cells into this space has been verified by electron microscopic studies (Farquhar 1961, Sano 1963 Herlant 1963 Maillard 1963, Weiss 1963)

Different opinions have been presented concerning the presence of mast cells in the pituitary gland Consolandi and Briziarelli (1952) have found consistently mast cells in human material and in addition a macro form, which they call *Micromastzellen* In a recent communication of Garvie (1963) very few mast cells were found in the anterior pituitary gland of the rat

## OWN INVESTIGATIONS

### Material and methods

The material consisted of adult Long Evans rats weighing 180—260 g at the beginning of the experiment they were subjected to conditions which are known to change the physiological state and histological picture of the adenohypophysis These included male rats which were castrated adrenalectomized, injected with testosterone (1 mg/day Neo Hombreol<sup>®</sup> Organon), cortisone (1 mg/day Adrenon<sup>®</sup> Organon), fed with thyroxine (0.2% mixed in food Thyranon<sup>®</sup> Organon) or with methylthiouracil (MTU 2% mixed in food 4(6) methylthiouracil Fluka AG) The experimental conditions of female rats were as follows: castration injections of estrogen (0.1 mg/day Dimenformon<sup>®</sup>, Organon), progesterone (1 mg/day Progestin<sup>®</sup> Organon) or reserpine (0.05 mg/day Serpasil<sup>®</sup>, Ciba) pregnancy (from 12th to 21st day) lactation (from 3rd to 10th day), 12 hours after discontinuation of lactation and immature female rats

The experimental conditions lasted 15, 30 and 60 days Each group consisted 6 rats, 2 animals were killed at each of the three time periods and their pituitary glands were removed immediately and fixed for 8 hours in modified Bouin Hollande fixative as presented by Herlant (1960a)

After deparaffinization the sections were treated for 3 min in Lugol's solution and then in 5% sodium thiosulphate solution in order to remove mercury salts. The



following staining methods were used: periodic acid Schiff orange G (Pearse 1949, 1950, 1961), aldehyde fuchsin (Halmi 1952a), alcian blue at pH 3.0 and pH 0.2 (Herlant 1960a), tetrachrome method (Herlant 1960a), acid fuchsin aniline blue (Fisher and Bulmer 1964), aldehyde thionin (Ezrin and Murray 1963) and azocarmine orange G (Dawson and Friedgood 1938).

## Results

### *Characteristics of the parenchymal cell types*

The identification of the different pituitary cell types was based on the findings obtained by using the different staining techniques on successive histological sections. The tentative identification was confirmed by recording specific changes in different cell types during the experimental conditions. The tinctorial characteristics of the cells thus became evident and are presented in Table II. In the following, different cell types are separately described. The characteristics of the cells are mainly based on the staining method which gave the best differentiation.

*Somatotropic cell* — The most abundant chromophilic cell type in the pituitary gland of the control rats and young immature rats was a small round or oval cell which was strongly orangeophilic in sections stained with methods which included orange G. In the azocarmine orange G method this cell type acquired ochre or light brown coloring (Fig. 1). This cell type is quite uniformly distributed throughout the whole anterior pituitary gland. Sometimes these cells are found in small groups or line along the sinusoids. Immature rats have an abundant number of these cells and during testosterone treatment these cells obtained an increased affinity for orange G. After methylthiourea feeding the intense staining of these cells was decreased and only some ochre stained granules were found in the cytoplasm and the cells look vesicular out. These somatotropic cells were intensely red stained in the acid fuchsin aniline blue method but could not be differentiated from the lactotropic cells in pregnant and lactating female rats. This differentiation could, however, be achieved by the tetrachrome method in addition to the azocarmine orange G method. Using the former method somatotropic cells were orange stained but in this method the concomitant aniline blue readily overstains these cells. Thus the latter method is more specific for the demonstration of somatotropic cells.

*Lactotropic cell* — Another cell type with orangeophilic properties in the PAS orange G staining method was present in augmented number during pregnancy, lactation and after treatment with estrogen. The azocarmine orange G method resulted in a chromophilic staining (Fig. 1).



following staining methods were used: periodic acid Schiff orange G (Pearse 1949, 1950, 1961), aldehyde fuchsin (Halmi 1952a), alcian blue at pH 3.0 and pH 0.2 (Herlant 1960a), tetrachrome method (Herlant 1960a), acid fuchsin aniline blue (Fisher and Bulmer 1964), aldehyde thionin (Exrin and Murray 1963) and azocarmine orange G (Dawson and Friedgood 1938).

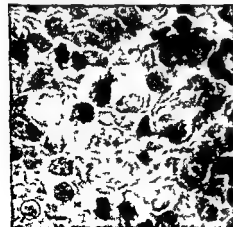
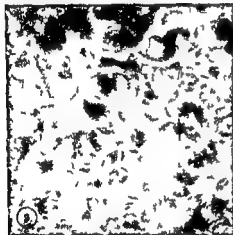
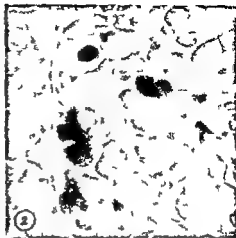
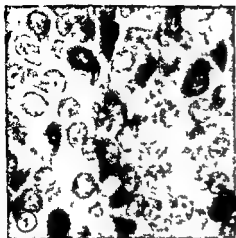
## Results

### *Characteristics of the parenchymal cell types*

The identification of the different pituitary cell types was based on the findings obtained by using the different staining techniques on successive histological sections. The tentative identification was confirmed by recording specific changes in different cell types during the experimental conditions. The tinctorial characteristics of the cells thus became evident and are presented in Table II. In the following different cell types are separately described. The characteristics of the cells are mainly based on the staining method which gave the best differentiation.

**Somatotropic cell** — The most abundant chromophilic cell type in the pituitary gland of the control rats and young immature rats was a small round or oval cell which was strongly orangeophilic in sections stained with methods which included orange G. In the azocarmine orange G method this cell type required ochre or light brown coloring (Fig 1). This cell type is quite uniformly distributed throughout the whole anterior pituitary gland. Sometimes these cells are found in small groups or line along the sinusoids. Immature rats have an abundant number of these cells and during testosterone treatment these cells obtained an increased affinity for orange G. After methylthiourea feeding the intense staining of these cells was decreased and only some ochre stained granules were found in the cytoplasm and the cells look vesicular out. These somatotropic cells were intensely red stained in the acid fuchsin aniline blue method but could not be differentiated from the lactotropic cells in pregnant and lactating female rats. This differentiation could, however, be achieved by the tetrachrome method in addition to the azocarmine orange G method. Using the former method somatotropic cells were orange stained but in this method the concomitant aniline blue readily overstains these cells. Thus the latter method is more specific for the demonstration of somatotropic cells.

**Lactotropic cell** — Another cell type with orangeophilic properties in the PAS orange G staining method was present in augmented number during pregnancy, lactation and after treatment with estrogen. The azocarmine orange G method resulted in a chromophilic staining (Fig 1).



*Thyrotropic cell* — The identification of the thyrotropic cells by the present methods was quite easy. With aldehyde fuchsin only certain central polygonal cells are stained red. The same cells are dark blue when the aldehyde thionin method was used (Fig 4). Alcian blue at pH 0.2 stained these cells green, while at pH 3.0 also other cells had some though weaker affinity for this dye (Fig 6). These AF positive cells are quite large cells, contain fine granulation and are not intimately bordering the sinusoids, but come into contact with these by their branches. Sometimes the cells are arranged in a grape like fashion along a sinusoid. After methylthiourea feeding, a greatly increased number of these cells was observed and also small irregular groups of these cells were present. Thyroxin treatment greatly diminished the number of the TSH cells and those found were shrunken.

TSH cells had a strong affinity for PAS staining. Using acid fuchsin, aniline blue as well as tetrachrome methods, the intensity of the blue stain in these cells was stronger than in other 'mucoid' cells.

*FSH cell* — FSH cells are quite similarly stained by the present methods with the gonadotropic cells which secrete LH. The methods by which differentiation can be obtained are the alcian blue method at pH 0.2 and the aldehyde thionin method. In the former, FSH cells are stained violet due to the affinity of these cells for alcian blue and PAS staining, while the latter component stains only LH cells. In the azocarmine orange G method the FSH cells are of somewhat violet hue due to small granules (Fig 5). The distribution of FSH cells in the rat pituitary gland was characteristic. They are mostly peripheral in the ventral part of the gland, but a large group of cells was present in the 'sex' zone, adjacent to the intermediate lobe. FSH cells are round or oval in shape, have a great affinity for PAS and are grouped around the sinusoids. The granules are coarse, but leave a round Golgi zone free. After castration a marked enlargement of these cells takes place and, after a longer time, vacuoles appear in the cytoplasm. Thirty days after castration some typical castration cells are formed with signet ring appearance. In young rats at about 30 days of age a large number of FSH cells was present on the ventro-caudal border of the pituitary gland.

*LH or ICSH cell* — LH cells are quite few in the normal male and female rat but after castration and during pregnancy a large number of cells appear which have a great affinity for PAS staining. At pH 0.2 these cells have only weak affinity for alcian blue and remain red due to the PAS staining. At pH 3.0 these cells are, however, light green colored (Fig 6). These cells are centrally situated but are easily separable from

the TSH cells by their round or oval shape and lighter staining affinity in methods like tetrachrome acid fuchsin aniline blue and azocarmine-orange G. The glycoprotein granulation is finer than that of FSH-cells. These cells are also intimately connected to the sinusoids but are not arranged in larger groups.

### *Other cell constituents of the adenohypophysis*

The capillary endothelial cells are somewhat PAS positive and stain red in the PAS orange G and alcian blue pH 3.0 methods. By the acid fuchsin aniline blue method these cells remain almost unstained but small fusiform cells in the perisinusoidal space are strongly blue stained. These perisinusoidal cells as well as capillary endothelial cells are blue both in azocarmine orange G, tetrachrome and aldehyde thionin methods.

Another cellular component which is intimately connected to the anterior pituitary tissue are the two sheets of cells lining the pituitary cleft between the anterior and intermediate lobes. The posterior epithelium covers the cleft on the side of the intermediate lobe and the anterior epithelium separates the anterior pituitary cells from the cleft. The latter cell line follows the anterior lobe when this is separated mechanically from the other lobes.

The posterior epithelium varies in different places: it is sometimes cubical, columnar or even stratified especially at the angles of the anterior and intermediate lobes and in places where blood vessels pierce the intermediate lobe. Differences in the height of the posterior epithelium has been recorded earlier in different experimental conditions, thus adrenalectomy and gonadectomy causes a flattening of the epithelium (Ferrer 1956). PAS positive goblet cells are also found between the epithelial cells.

The anterior epithelium is not continuous. Both acidophilic and basophilic cells push between the epithelial cells into a connection with the cleft lumen. Increased basophilic cell penetration is found after castration and the basophilic cells in question seem to be FSH gonadotrophs. The colloid content in the cleft is much increased after castration but can also be found in control animals and after adrenalectomy. Young animals and those receiving estrogen, testosterone or cortisone are devoid of colloid. When a large amount of colloid is present both the anterior and the posterior epithelial linings are decreased in height. Vacuoles of different size can then be seen in colloid close to the epithelial cells. The colloid in the pituitary cleft stains red with PAS staining, violet red with aldehyde

thionin and blue with azocarmine orange G. Within the colloid, sometimes fragments of anterior pituitary basophils or acidophils can be found together with some erythrocytes.

## Discussion

The simultaneous demonstration of all the different pituitary cell types by a single staining method appeared to be impossible. Thus a combination of different staining techniques was used in order to reveal specifically a certain cell type. The topographic distribution pattern of cells in the pituitary gland serves as a further aid in the identification of the cells.

Concerning the cellular site of STH, LTH and gonadotropic hormone production the present results are in agreement with most of the recent reports in the literature. Of the two different groups of gonadotrophs the one (FSH cells) is mainly peripheral while the other (LH cells) is in the central part of the lobe, the latter differs somewhat from the thyrotropic cells in shape and distribution rendering differentiation possible. Best differentiation between FSH and LH cells was obtained by the alcian blue technique at pH 0.2. In other staining methods these cells were stained about equally. The affinity of the granules of these gonadotropic cells to basic dyes as well as to alcian blue at higher pH and basic fuchsin in Schiff staining was clearly weaker than that of the thyrotropic cells.

Except on the basis of the differential staining characteristics the two acidophilic cell types the somatotropic and the LTH cells can also be identified due to their typical changes in various experimental conditions as described earlier.

Estrogen treatment produces a great enlargement of the pituitary gland. It has been found that the acidophilic cell group is activated by this treatment (Severinghaus 1939; Balcer and Everett 1944; Meyer et al 1946). Purves and Giesbach (1952) identified the LTH cells as the acidophils which degranulate after castration and estrogen treatment. Quantitative studies have shown a marked increase of the LTH content in the pituitary gland after estrogen treatment (Reece and Turner 1937; Meites and Turner 1948) and the same is found during lactation (Hurst and Turner 1942). LTH cells were few in normal male animals but were increased after castration, a fact described also earlier (Hurst and Turner 1942; Lacour 1950; Dubois and Girod 1964). If a female mouse is castrated before sexual maturation the LTH cells fail to differentiate (Barnes 1962).

The site of corticotropic hormone production in the pituitary gland of

the rat is a very disputed subject and the demonstration of the possible cell type demands increased ACTH formation and secretion with the corresponding changes in cellular morphology. The changes are not however always appropriate evidence for the origin of a certain hormone. This is confirmed e.g. by the marked decrease of acidophilic cells after thyroidectomy or methylthiouracil feeding although it is well known that these cells do not take part in the production of TSH (Griesbach 1953). The cell type which in this study is suggested to be the ACTH cell greatly resembles that described by Herlant and his colleagues (Ricadot and Herlant 1960, Pasteels and Herlant 1962, Herlant and Klaterski 1963, Quenum and Herlant 1964).

Quite specific staining techniques for the thyrotropic cells seem to be aldehyde fuchsin and aldehyde thionin methods. It was found, however, that the dye easily deteriorated during storage although it was stored at room temperature as recommended by Elftman (1959). Although the TSH cells were not stainable the aged dye solution was still capable of staining the neurosecretory material in the posterior lobe. Identification of these cells was verified through the marked changes in the number of the cells found after the treatment with thyroxine or methylthiouracil as reported previously (Zeckwer et al 1935, Catchpole 1949, Purves and Griesbach 1951b, Halmi 1952b, Dhoni and Tietze 1962, Dhoni et al 1962).



# HISTOCHEMISTRY OF CARBOXYLIC ESTERASES IN THE ADENOHYPOPHYSIS

## LITERATURE REVIEW

### Classification and characterization of carboxylic esterases

The *cholinester hydrolases* as an esterase group represent a highly inhomogeneous group of enzymes and many different variants have been reported (cf Svensmark 1965). Two types of *cholinesterases* (ChE) can be rather specifically separated: acetylcholinesterase or acetylcholine acetyl hydrolase (AChE) and non specific cholinesterase or acylcholine acyl hydrolase (ns ChE). Both types of these can be separated from non specific esterases (ns E) due to their susceptibility to eserine at low concentration ( $10^{-4}$  M). AChE and ns ChE differ from each other in substrate specificity and in the inhibitory characteristics with respect to different organophosphate esters, carbamate derivatives and quaternary ammonium salts (Aldridge 1963a, Augustinsson 1960, Igaras 1961).

The distinction of *lipase* (glycerol ester hydrolase) from the other ns Es is based on its ability to act upon esters of long chained fatty acids (C8 and upwards) or on undigested substrates and on the specific activation by bile acids. Cholesterol esterase (Myers et al 1955) and a lipase in lung and adipose tissue (Hulsmann et al 1964) have been differentiated from the pancreatic lipase.

The *non specific esterases* (ns E) which hydrolyze short chained fatty acid esters were classified by Aldridge (1953b, c) into organophosphorous resistant (A esterases, E r ns E) and sensitive (B esterases E s ns E) groups, the most used inhibitor for this distinction has been diethyl p nitrophenylphosphate (E600). A third type of enzyme called *C esterase* is differentiated from the E600 resistant group by its inability to hydrolyze organophosphorous compounds and by the activation of the hydrolysis with low concentrations of p chloromercuribenzoate (p CMB, Bergmann et al 1957, Bergmann and Pimon 1959, 1960). Gomori (1955) made further distinction in the E600 sensitive group on the basis of the response to NaF and called the NaF resistant enzymes the pancreatic type and the NaF sensitive the hepatic type of esterase.

A large number of *proteases* and *peptidases* have been described which are capable of hydrolyzing in addition to peptide bonds esters of substituted amino acids and peptides (Neurath and Schwert 1950, Wiggans et al. 1954, Johnston 1956, Myers et al 1957, Martin and Axelrod 1957, Shippey and Binklev 1958, Lagunoff and Benditt 1964). It has been concluded that the esterases especially E600 resistant forms as revealed by histochemical techniques may in fact include different intracellular proteases or

peptidases such as cathepsin C (Hess and Pearse 1958) It has become evident later however that the latter enzyme is distinct from any esterase activities hydrolyzing  $\alpha$ -bromonaphthyl acetate one of the most used substrates for E<sub>1</sub> and E<sub>2</sub> (Vanha Perttula et al 1965) Some trypsin like (Glennner et al 1963 Hopsu and Glennner 1963a, b) and chymotrypsin like (Lagunoff and Benditt 1964, Riekkinen and Hopsu 1965) enzymes as well as acylases in different tissues (Hopsu and Glennner 1964 Hopsu et al 1965a, b) have been suggested to contribute to the hydrolysis of various naphthol esters

A variant of carbonic anhydrase has been described which is capable of hydrolyzing ester substrates, and a tentative name of D esterase has been suggested for it (Shaw et al 1960 Tappan et al 1961) Electrophoretic separation of carbonic anhydrase isozymes from esterase activities has been however reported in erythrocyte hemolysates and rat kidney extracts (Hirvippa et al 1966)

## Adenohypophyseal carboxylic esterases

### Cholinesterases

In the human pituitary gland no ChE was found localized mainly in fully granulated basophils but some activity was also present in chromophobes and in the colloid (Pearse 1952) The existence of AChE in the FSH cells of the pituitary gland of rabbit was shown by Dumont (1956a), no AChE activity could be demonstrated In the guinea pig pituitary gland, on the other hand ChE activity was mostly non specific (Dumont 1956b) This activity was also located in beta basophils (FSH cells) but butyrylthiocholine was also hydrolyzed in extracellular colloid and capillary endothelial cells

Differences have been observed in the content of AChE and no ChE in different parts of the pituitary gland of different animals (Loelle and Geesey 1961 Arvy 1961, 1962 1964, Arvy and du Mesnil du Buisson 1961 Kobayashi and Farner 1964) As a rule however both ChEs are located mainly in the paramedian zone of the anterior pituitary gland in mammals and in the cephalic part in the gland of the sparrow With biochemical methods only AChE was demonstrated in the anterior and posterior pituitary lobes of the beef (Pasetto 1958)

### Non specific esterases

The first demonstration of carboxylic esterase activity in the anterior pituitary tissue of the rat was made by Ledu and Wislocki (1952) using  $\alpha$ -naphthyl acetate as substrate Using the tween technique of Gomori (1945) for the demonstration of esterase and lipase activity no hydrolysis was found in the rat pituitary gland by Verne and Hébert (1952) and Verne (1954) In human pituitary gland the same method gave a distribution which suggested that the lipase activity may be in close connection to the pituitary lipids and have some effect on the permeability of the cell membranes (Curri 1956) Rat adenohypophysis was classified as an organ of slight esterolytic activity toward  $\alpha$ -bromonaphthyl acetate by Barnett (1955)

Gomori (1955) found that anterior pituitary basophils contained an esterase activity which can be revealed using  $\alpha$ -naphthyl acetate  $\alpha$ -naphthyl propionate  $\alpha$ -naphthyl acrylate  $\alpha$ -naphthyl butyrate or naphthol AS acetate as substrates The activity is

totally inhibited by E600 ( $10^{-4}$ M), markedly inhibited by diisopropyl fluorophosphate (DFP,  $10^{-4}$ M), but not inhibited with NaF ( $0.5-2 \times 10^{-2}$ M), it was slightly activated by lauryl sulfate

Esterase activity was found in human pituitary basophilic cells by Fand (1955) after paraffin embedding. She however, could show that there is a group of basophils in the intermediate lobe and invading the posterior lobe which were devoid of  $\alpha$ -naphthyl acetate and naphthol AS acetate splitting activity. Both cell types could be stained with PAS and aldehyde fuchsin. Further studies on human material (Fand 1961) have demonstrated that the enzymatically active basophils of the anterior pituitary gland may be gonadotrophs and such cells are sometimes also invading the posterior lobe, especially in females.

Further studies on human material showed the existence of two types of esterase which were resistant to eserine (Pearse 1956). One of them was located in the PAS positive "mucoid" cells. This activity was sensitive to E600 ( $10^{-4}$ M) and was thus classified as B esterase. It was observed with acetate and butyrate esters of indoxyl as well as with  $\alpha$ -naphthyl acetate and naphthol AS acetate but not with indoxyl laurate. These findings agree with those of Gomori (1955) in respect to the pancreatic type of hypophyseal esterase. The possibility of the existence of two nearly identical enzyme activities in mucoid cells was suspected on the basis of different results obtained with fresh frozen sections and after dehydration and paraffin embedding. The same group of basophils found by Fand (1955) to lack esterase activity was active in fresh frozen sections but could not be found after paraffin embedding. It was suggested that the enzyme may occur in two different forms of which one was lost during the dehydration procedure.

NaE activity with different substrate and inhibitor characteristics was found in perisinusoidal cells (Pearse 1956). This enzyme activity utilizes only acetate esters of naphthol and indoxyl but not esters with longer chain length. It increases during pregnancy and in other states with increased degranulation of basophilic "mucoid" cells. It was suggested that this esterolytic activity may be caused by an intracellular peptidase.

In rat pituitary gland naE activity was histochemically demonstrated by Lojda (1960) and Lojda and Schreiber (1960, 1964). With  $\alpha$ -naphthyl acetate and naphthol AS acetate as substrate maximum activity was observed in gonadotropic and thyrotropic cells. Both E<sub>s</sub> naE and E<sub>r</sub> naE activities were present in these cells.

Using 5-bromoindoxyl acetate as substrate Herlant and Grignon (1961) found esterase activity in the turtle pituitary gland in two different cell types: somatotrophic and lactotrophic cells. This activity was thought to be associated with the high phospholipid content of these two cell types. An E<sub>r</sub> naE has also been demonstrated in gonadotropic cells of various amphibian species (Wachtler and Pearse 1966).

Sobel (1962) observed that thyrotropic cells of the rat pituitary gland contained a diffuse cytoplasmic esterase as well as an E<sub>r</sub> naE located identically with acid phosphatase probably in the lysosomal particles and in addition in the Golgi zone. This activity was thought to be associated to the secretion of thyrotropic hormone.

Studies on human embryos have shown that no esterase activity is present in Rathke's pouch either in 6 and 7 mm long embryos (McKay et al. 1956) or 45 mm long embryos (Posn et al. 1954, 1957). Pearse (1956) suggested that both thyrotrophs and gonadotrophs are active in the fetal gland, the first appearance of the enzyme activity coincided with the beginning of the mucoprotein hormone secretion of these cells and

the enzyme activity was associated with the mucoprotein granules Jirásek (1963) has also studied the esterase activity in the fetal pituitary gland of man and could differentiate two different cell types which were active from the end of the third month of development. In PAS positive peripheral and central cells an enzyme activity appears which is also found in paraffin embedded sections. From the fourth month on another activity in chromophobe cells of the basophilic line became active but this activity was present only in fresh frozen sections. These cells showed perinuclear activity and, in addition, there was a diffuse cytoplasmic reaction. Cells covering the rest follicles in the intermediate lobe showed also an esterase activity in the apical part of the cytoplasm.

In addition to the basophils esterase activity has also been demonstrated in other hypophyseal cell types. Thus Pearce (1956) demonstrates another esterase activity in perinuclear cells. This enzyme activity corresponds to that found in pericytic cells and neurons of hypothalamic nuclei (Pepler and Pearce 1957, Pearce 1958) and in some cellular components of rat kidney (Hees and Pearce 1958). On the basis of activator and inhibitor studies it was suggested that this may be an intracellular peptidase probably cathepsin C.

The cellular distribution of Esterase is mainly diffuse or finely granular in basophilic and PAS positive cells in different animal species including man (Gomori 1955, Fand 1956, 1961, Pearce 1956, Sobel 1962, Jirasek 1963, Loyda and Schreiber 1960, 1964). Esterase has been found to reside near the Golgi zone (Pearse 1956, Sobel 1962, Jirasek 1963, Loyda and Schreiber 1960, 1964). This activity is the only one observed after paraffin embedding (Pearse 1956) and it has been claimed that this activity is confined to small corpuseles which may be lysosomes (Pearse 1956, Pepler and Pearce 1957, Hees and Pearce 1958, Pearce 1961, Pearce and van Noorden 1963a, b, Sobel 1962, Loyda and Schreiber 1964, Wachtler and Pearce 1966).

## Changes in experimental conditions

No reports have been published of variations in the ChE activity of the anterior pituitary cells in experimental conditions. In human material the diffuse cytoplasmic Esterase activity follows the 'excretory pattern' of the cells (Pearse 1956). During the increased activity of the 'mucoid' cells the esterase activity is moved to the cellular periphery near the bordering perinuclear space. Simultaneously increased activity is observed in pericytes and transfer of esterase to these cells by phagocytosis was suggested. Studies of Jirásek (1963) do not confirm the increased esterase activity in human adenohypophysis in connection with the removal of the PAS positive granula from the cells but on the contrary increased esterase activity is connected with the synthesis of PAS positive glyco- and mucoprotein granules.

In man increased granular perinuclear activity was found during pregnancy in acidophils, thyroidectomy cells had also the same type of activity (Pearse 1956). Thyroidectomy and feeding of methylthiouracil in rats increases the granular Esterase activity (Pearse 1956, Sobel 1962, Loyda and Schreiber 1960, 1964). Simultaneously a decrease in enzyme activity in other 'mucoid' cells as well as a very marked increase in 'mucoid' cells of the pars intermedia was reported (Pearse 1956).

Castration cells in rats have also been found to contain increased amount of both

totally inhibited by E600 ( $10^{-4}M$ ), markedly inhibited by diisopropyl fluorophosphate (DFP,  $10^{-4}M$ ), but not inhibited with NaF ( $0.5-2 \times 10^{-3}M$ ), it was slightly activated by lauryl sulfate

Esterase activity was found in human pituitary basophilic cells by Fand (1955) after paraffin embedding. She, however, could show that there is a group of basophils in the intermediate lobe and invading the posterior lobe which were devoid of  $\alpha$ -naphthyl acetate and naphthol AS acetate splitting activity. Both cell types could be stained with PAS and aldehyde fuchsin. Further studies on human material (Fand 1961) have demonstrated that the enzymatically active basophils of the anterior pituitary gland may be gonadotrophs and such cells are sometimes also invading the posterior lobe, especially in females.

Further studies on human material showed the existence of two types of esterase which were resistant to eserine (Pearse 1956). One of them was located in the PAS positive 'mucoid' cells. This activity was sensitive to E600 ( $10^{-4}M$ ) and was thus classified as B esterase. It was observed with acetate and butyrate esters of indoxyl as well as with  $\alpha$ -naphthyl acetate and naphthol AS acetate but not with indoxyl laurate. These findings agree with those of Gomori (1955) in respect to the pancreatic type of hypophyseal esterase. The possibility of the existence of two nearly identical enzyme activities in mucoid cells was suspected on the basis of different results obtained with fresh frozen sections and after dehydration and paraffin embedding. The same group of basophils found by Fand (1955) to lack esterase activity was active in fresh frozen sections but could not be found after paraffin embedding. It was suggested that the enzyme may occur in two different forms of which one was lost during the dehydration procedure.

As E activity with different substrate and inhibitor characteristics was found in perisinusoidal cells (Pearse 1956). This enzyme activity utilizes only acetate esters of naphthol and indoxyl, but not esters with longer chain length. It increases during pregnancy and in other states with increased degranulation of basophilic 'mucoid' cells. It was suggested that this esterolytic activity may be caused by an intracellular peptidase.

In rat pituitary gland as E activity was histochemically demonstrated by Lojda (1960) and Lojda and Schreiber (1960, 1964). With  $\alpha$ -naphthyl acetate and naphthol AS acetate as substrate maximum activity was observed in gonadotropic and thyrotropic cells. Both E<sub>1</sub> as E and E<sub>2</sub> as E activities were present in these cells.

Using  $\beta$ -bromoindoxyl acetate as substrate Herlant and Grignon (1961) found esterase activity in the turtle pituitary gland in two different cell types: somatotropic and lactotropic cells. This activity was thought to be associated with the high phospholipid content of these two cell types. An E<sub>1</sub> as E has also been demonstrated in gonadotropic cells of various amphibian species (Wächter and Pearse 1956).

Sobell (1962) observed that thyrotropic cells of the rat pituitary gland contained a diffuse cytoplasmic esterase as well as an E<sub>2</sub> as E located identically with acid phosphatase probably in the lysosomal particles and in addition in the Golgi zone. This activity was thought to be associated to the secretion of thyrotropic hormone.

Studies on human embryos have shown that no esterase activity is present in Rathke's pouch either in 6 and 7 mm long embryos (McKay et al 1956) or 4.5 mm long embryos (Rosen et al 1954, 1957). Pearse (1956) suggested that both thyrotrophs and gonadotrophs are active in the fetal gland, the first appearance of the enzyme activity coincided with the beginning of the mucoprotein hormone secretion of these cells and

and they were all obtained from Sigma Chem Comp (Ohio USA). The final concentration of the substrate was  $\mu$  M. Fast Garnet CBC (Edward Gurr Ltd London England) was used as coupling agent at a concentration of 8 mg/ml and no counter stain was used. The optimal incubation times for the different substrates were as follows:  $\alpha$  NA,  $\alpha$  NPr and N AS for 3–5 min;  $\alpha$  NB and  $\alpha$  NV for 6–10 min at room temperature and  $\alpha$  NC, N ASD and  $\beta$  NL for 30–60 min at 37°C. When inhibitors were used 30 min preincubation at room temperature or at 37°C respectively preceded without substrate and coupling agent. The inhibitor substances were also present at the same concentration in the final substrate solution.

■ Bromoindoxyl acetate (5 BIA) was used as substrate according to the method of Holt (1952, 1956–1958) and Holt and Withers (1958). The substrate solution was prepared according to Pearce (1961), but in addition the hydrolysis was tested at a lower (0.5 mM) ferri- and ferricyanide concentration (Shnitka and Seligman 1961). The incubation time was 30–60 min at room temperature. The method of Wachstein et al (1961) was employed for the hydrolysis of thioacetic acid (TAA Fluka AG Buchs SG Switzerland). The incubation time was 60 min at 37°C.

## Modifiers for the classification of esterases

The inhibitor substances used in the present investigation are listed in Table III where also the routinely used concentration of the inhibitor for any sensitive enzyme is given. Sodium taurocholate is also used to activate the hydrolysis of long chained fatty acid esters of  $\alpha$  and  $\beta$  naphthol by a pancreatic type of lipase (Abe et al 1964) possibly present in pituitary tissue.

Other modifier substances used in the studies were as follows: Sodium 1-chloromercuribenzoate (p CMB Calbiochem Calif USA) at a concentration of  $10^{-4}$  M was used with E600 ( $10^{-4}$  M) to study the presence of C type esterase activity (Bergmann et al 1951; Bergmann and Rimón 1953–1960). The effects of Triton X 100 (Mitar Lautenbourg France) on the histochemical reaction was tested at concentrations of 0.05 and 0.1% (Allen et al 1965). Perazine (Perazine<sup>®</sup>, Laake OY, Turku Finland) was used at  $10^{-4}$  M concentration to inhibit phenothiazine sensitive esterases (Hulsman et al 1964).

## Results

### *Carboxylic esterases in normal rat pituitary sections*

The intensities of the esterase activities in the different pituitary cell types are presented in Tables IV and V as obtained with different substrate-inhibitor combinations. The intensities are expressed with signs + + + +, + + +, + +, +, and —, which mean the decreasing order of the enzymic reaction obtained. Table IV summarizes the results for fresh sections and Table V for formalin fixed sections.

Table III Inhibitors used in the differentiation and classification of esterases

Inhibitor	Abbreviation	Source	Concentration (M)	Sensitive enzymes	Reference
Pearline sulphate	Pearline	Nutr Biochem Corp Okla USA	10 <sup>-4</sup>	ChE	Aldridge 1953a Gomori 1952
1, 2 bis (4 trimethyl ammoniumphenyl) pentan-3 one diiodide	62 C-47	Wellcome Res Lab Beckenham, England	10 <sup>-4</sup>	ACHE	Bayliss & Todrick 1956 Pepler & Pearce 1957
1, 5 bis (4 allyl dimethyl ammoniumphenyl) pentan-3 one diiodide	284 C-51	Wellcome Res Lab Beckenham, England	10 <sup>-4</sup>	ACHE	Bayliss & Todrick 1956 Holmstedt 1957
Tetra isopropyl pyrophosphoramide	iso OMPA	Light & Co Ltd Colabrook England	10 <sup>-4</sup>	ns ChE	Aldridge 1953a Diegenbach 1953
Lithopropazine methosulphate	Lithovane	May & Baker Ltd Dagenham England	10 <sup>-4</sup>	ns ChE	Bayliss & Todrick 1956
Diethyl p nitrophenyl phosphate	E800	Mintacol® Bayer Leverkusen Germany	10 <sup>-4</sup>	Le ns F	Aldridge 1953a, b, c
Di isopropyl fluoro phosphonate	DFP	Light & Co Ltd Colabrook, England	10 <sup>-4</sup>	Es ns E	Aldridge 1953a, b, c
Sodium taurocholate	TO	British Drug Houses Poole, England	10 <sup>-4</sup>	ns E	Abe et al 1964
Sodium fluoride	NaF	E Merck AG Darmstadt, Germany	10 <sup>-4</sup>	Es ns E hepatic type	Gomori 1954
Cupric chloride	Cu	E Merck AG Darmstadt Germany	10 <sup>-4</sup>	Le ns F A esterase	Pearse 1956 Bergmann et al 1957

Table IV E terases in fresh frozen sections obtained with different substrate inhibitor combinations The inhibitor concentrations as given in Table III.

Substrate and inhibitor	Gonado troph	Thyro troph	Somato troph	Peri cytes	Capillary endo thelium	Cleft epi thelium
AcThC	—	—	—	—	+	+
150 OMPA	—	—	—	—	+	—
284 C 51	—	—	—	—	+	+
Eserine	—	—	—	—	+	—
E600	—	—	—	—	+	—
Ly riane	—	—	—	—	+	—
BuThC	—	—	—	—	+	+
150 OMPA	—	—	—	—	+	—
284 C 51	—	—	—	—	+	+
Eserine	—	—	—	—	+	—
E600	—	—	—	—	+	—
Ly riane	—	—	—	—	+	—
$\alpha$ N1	+++	+++	++	—	—	+
150 OMPA	+++	+++	++	—	—	—
284 C 51	+++	+++	++	—	—	+
Eserine	+++	+++	++	—	—	+
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	—
Cu <sup>++</sup>	++	++	+	—	—	+
p CMB	+++	+++	++	—	—	+
TC	+	+	++	—	—	+
$\alpha$ NPr	+++	+++	+	—	—	++
150 OMPA	+++	+++	+	—	—	++
284 C 51	+++	+++	+	—	—	++
Eserine	+++	+++	+	—	—	++
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	—
Cu <sup>++</sup>	++	++	+	—	—	+
p CMB	+++	+++	+	—	—	++
TC	+	+	+	—	—	+
$\alpha$ NB	+	+	±	—	—	+++
150 OMPA	+	+	±	—	—	+++
284 C 51	+	+	±	—	—	+++
Eserine	+	+	±	—	—	+++
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	—
Cu <sup>++</sup>	+	+	—	—	—	++
p CMB	+	+	±	—	—	+++
TC	±	±	—	—	—	++



Table IV (continued)

Substrate and inhibitor	Gonadotroph	Thyrotroph	Somatotroph	Pericytes	Capillary endothelium	Cleft epithelium
$\alpha$ NV	—	—	—	—	—	++
$\alpha$ NC	—	—	—	—	—	—
N AS	—	—	—	—	—	—
N ASD	—	—	—	—	—	—
5 BIA	+	+	+	—	—	—
180 OMI 1	+	+	+	—	—	—
284 C 51	+	+	+	—	—	—
Eserine	+	+	+	—	—	—
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	—
Cu <sup>+</sup>	—	—	—	—	—	—
p OMB	+	+	+	—	—	—
TC	—	—	—	—	—	—
TAA	—	—	—	—	—	—

## Hydrolysis of thiocholine esters

In *unfixed sections* the hydrolysis of both AcThC and BuThC was non-ly absent except for some weak activity in capillary endothelial cells and in cells lining the pituitary cleft after 24 hours of incubation.

In *fixed sections* both these activities were also present but additionally some activity was obtained in some large cells in the central part of the gland. These cells were polygonal (Figs 7 and 10) and clearly reminiscent of the TSH cells in histological studies. With both substrates the reaction product was mainly diffuse but small granules were also found. The reaction in the cleft epithelium was diffuse, but the weak activity in capillary endothelium seemed to be mainly granular (Figs 8 and 11). The intensity of these reactions was quite weak in comparison to the activities found in the posterior lobe. Long incubation times were necessary.

The hydrolysis of both substrates was totally inhibited in the polygonal cells by eserine (Fig 8), 180 OMPA (Fig 11) and E600 (Fig 12), but not by 284 C 51 (Fig 9). The diffuse activity in the cleft epithelium had identical characteristics which are typical for the *ns ChE*.

The weak granular activity following the sinusoids was resistant to eserine (Fig 8), 180 OMPA (Fig 11) as well as to E600 (Fig 12). Thus this reaction cannot be ascribed to either AChF or *ns ChE*.

Table I Esteraes in formalin fixed sections obtained with different substrate-inhibitor combinations. The inhibitor concentrations as given in Table III

Substrate and inhibitor	Gonadotroph	Thyrotroph	Somatotroph	Paraverte	Capillary endothelium	Cleft epithelium
AcTh	—	+	—	—	+	++
150-OMPA	—	—	—	—	+	—
284 C 51	—	+	—	—	+	++
Eserine	—	—	—	—	+	—
E600	—	—	—	—	+	—
Lysergic	—	—	—	—	+	—
BuThC	—	+	—	—	+	++
150 OMPA	—	—	—	—	+	—
284 C 51	—	+	—	—	+	++
Eserine	—	—	—	—	+	—
E600	—	—	—	—	+	—
Lysergic	—	—	—	—	+	—
$\alpha$ NA	++++	+++	++	+++	++	++
150 OMPA	++++	+++	++	+++	++	++
284 C 51	++++	+++	++	+++	++	++
Eserine	++++	+++	++	+++	++	++
E600	++	++	+	—	++	—
DFP	++	++	+	—	—	—
NaF	++	++	+	—	++	—
Cu	++	++	+	++	—	++
p-CMB	+++++	+++	++	++	++	++
TC	++++	+++	++	++	++	++
$\alpha$ NPr	++	++	+	+++	—	+++
150-OMPA	++	++	+	+++	—	+++
284 C 51	++	++	+	+++	—	+++
Eserine	++	++	+	+++	—	+++
E600	+	+	—	—	—	—
DFP	+	+	—	—	—	—
NaF	+	+	—	—	—	—
Cu <sup>++</sup>	+	+	+	++	—	+++
p-CMB	++	++	+	+++	—	+++
TC	++	++	+	+++	—	+++
$\alpha$ NB	+	+	—	++++	—	++++
150-OMPA	+	+	—	++++	—	++++
284 C 51	+	+	—	++++	—	++++
Eserine	+	+	—	++++	—	++++
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	+
Cu <sup>++</sup>	+	+	—	++++	—	++++
p-CMB	+	+	—	++++	—	++++
TC	+	+	—	++++	—	++++

Table IV (continued)

Substrate and inhibitor	Gonadotroph	Thyrotroph	Somatotroph	Pericytes	Capillary endothelium	Cleft epithelium
$\alpha$ NV	—	—	—	—	—	++
$\alpha$ NC	—	—	—	—	—	—
NAS	—	—	—	—	—	—
NASD	—	—	—	—	—	—
5 BIA	+	+	+	—	—	—
iso OMPA	+	+	+	—	—	—
294 C 51	+	+	+	—	—	—
Eserine	+	+	+	—	—	—
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	—
Cu <sup>++</sup>	—	—	—	—	—	—
p CMB	+	+	+	—	—	—
TC	—	—	—	—	—	—
TAA	—	—	—	—	—	—

### Hydrolysis of thiocholine esters

In *unfixed sections* the hydrolysis of both AcThC and BuThC was nearly absent except for some weak activity in capillary endothelial cells and in cells lining the pituitary cleft after 24 hours of incubation.

In *fixed sections* both these activities were also present, but additionally some activity was obtained in some large cells in the central part of the gland. These cells were polygonal (Figs 7 and 10) and clearly reminiscent of the TSH cells in histological studies. With both substrates the reaction product was mainly diffuse but small granules were also found. The reaction in the cleft epithelium was diffuse but the weak activity in capillary endothelium seemed to be mainly granular (Figs 8 and 11). The intensity of these reactions was quite weak in comparison to the activities found in the posterior lobe; long incubation times were necessary.

The hydrolysis of both substrates was totally inhibited in the polygonal cells by eserine (Fig 6), iso OMPA (Fig 11) and E600 (Fig 12) but not by 294 C 51 (Fig 9). The diffuse activity in the cleft epithelium had identical characteristics which are typical for the ns ChE.

The weak granular activity following the sinusoids was resistant to eserine (Fig 8), iso OMPA (Fig 11) as well as to E600 (Fig 12). Thus this reaction cannot be ascribed to either AChE or ns ChE.



- 7 AcThC Fixed section  $\times 240$   
 8 AcThC Fixed section Eserine 10 M  $\times 240$   
 9 AcThC Fixed section 284 C 51 10 M  $\times 240$   
 10 BuThC Fixed section  $\times 240$   
 11 BuThC Fixed section Iso OMPA 10 M  $\times 240$   
 12 BuThC Fixed section EtOO 10 M  $\times 240$   
 13 a NA. Unfixed section  $\times 150$   
 14 a NA. Unfixed section Thioth  $\times 100$  2.25 ml 100% EtOH

clearly diminished activity was present in these cells after sodium turocholate treatment (Fig 15)

In addition to these cells a weaker activity was found in almost all other cells. Finely granular deposits of the reaction product was in the cytoplasm increasing towards the cell periphery. The overall result in the histological section was a net or honeycomb like picture (Figs 13 and 15). This type of activity is most clearly present in somatotrophic cells and it has the same affector characteristics as those previously reported for esterase activity in gonadotropic and thyrotrophic cells. When Triton X 100 was included in the incubation medium at 0.1 % concentration no enzymic activity was found in the anterior pituitary cells. At 0.05 % concentration the gonadotropic and thyrotrophic cells were still devoid of activity, but an intense net like activity was shown corresponding mainly with the cellular periphery of the somatotrophic cells (Fig 14), under which it is also found in Table IV and V. The same activity was also quite well preserved in the presence of turocholate (Fig 15).

The epithelium covering the residual cleft between anterior and intermediate lobes showed some activity with the same affector characteristics as the former cell types (Fig 49 black arrow). Triton X 100 (0.05 %) and sodium turocholate were only slightly inhibitory. The activity with  $\alpha$ -N as substrate was however rather weak in comparison to some other substrates reported later.

In *formalin fixed sections* a very intense esterase activity was obtained after a much shorter incubation time than that used in fresh frozen sections. This indicated that at least some of the esterase activities were soluble and could not be revealed in unfixed sections. This was further confirmed by the inhibitor studies.

The most active cells turned out to be again the gonadotropic FSH and LH cells, but the polygonal thyrotrophic cells showed also an intense activity. The activity was located in these cells near the nucleus and the reaction appeared first as coarse granules. After a longer incubation time the reaction products tended to cover also the nuclear area (Fig 16). This activity was clearly augmented by pCMB (Fig 17). P600 and DFP were without effect on this coarse granular activity.

An L-rns P activity was also to a smaller degree observed in numerous other cell types. The most active cells were somatotrophic cells, but ACTH cells also showed this type of esterase. The reaction was granular and most active perinuclearly. No differences were observed in the inhibitor studies in respect to the coarse granular activity in gonadotropic and thyrotrophic cells.

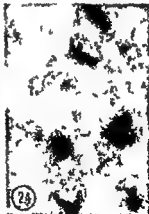
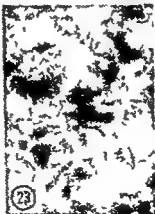
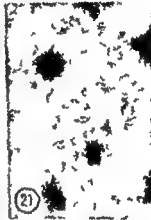
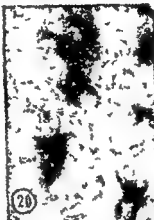
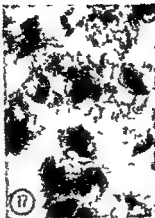
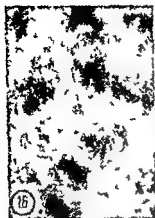


Fig 16  $\alpha$  NA Fixed section  $\times 40$

Fig 17  $\alpha$  NA Fixed section  $\times 240$

Fig 18  $\alpha$  NA Fixed section  $\times 240$

Fig 19  $\alpha$  NA Fixed section  $\times 240$

Fig 20  $\alpha$  NA Fixed section  $\times 240$

Fig 21  $\alpha$  NA Fixed section  $\times 240$

Fig 22  $\alpha$  NA Fixed section  $\times 240$

Fig 23  $\alpha$  NA Fixed section  $\times 240$

Fig 24  $\alpha$  NA Fixed section  $\times 240$

The sinusoidal epithelial cells also consistently showed a fine granular I<sub>1</sub> activity. This differed from the other I<sub>1</sub> activities due to its sensitivity to DPP and Cu<sup>+</sup> (Fig. 18). The epithelial cells of the residual cleft showed more intense activity in fixed sections than in fresh frozen sections. This activity had the same inhibitor characteristics as described and thus seems to represent an I<sub>1</sub> activity. The other I<sub>1</sub> activities found in fresh frozen sections cannot be observed in fixed sections due mainly to their inactivation in the fixative.

Another esterolytic activity was revealed which was not evident in unfixed sections. This activity resides in fusiform cells around the sinusoidal capillaries. This cell corresponds in its appearance and location to the perisinusoidal cell. The enzymically active cells in the normal rat were preferentially located in areas with numerous active GSII cells, i.e. in the sex zone. This activity was sensitive to F600, DPP and NaF, it thus exhibited the same characteristics as the esterase in fresh sections. Triton X 100 at 0.05 and 0.1% concentration had some activating effect on this esterase. Cu<sup>+</sup> does not inhibit this enzyme and the active pericytic cells are thus differentiated from capillary endothelial cells which have Cu<sup>+</sup> sensitive activity (Fig. 18).

*$\alpha$ -Naphthyl propionate* —  $\alpha$ -NPr revealed nearly identical distribution and inhibitor characteristics of hydrolytic activity in *fresh frozen sections* as  $\alpha$ -NA. The results obtained with Triton X 100 are also in agreement with the findings obtained with  $\alpha$ -NA. All of these characteristics suggest that  $\alpha$ -NA and  $\alpha$ -NPr are hydrolyzed by the same enzymes. Some relative intensity differences between the different sites of activity were however observed. Thus the honeycomb-like distribution of hydrolytic activity turned out to be weaker in comparison to the esterase activity in gonadotropic and thyrotropic cells (Fig. 19), while, on the contrary, a much more appreciable activity was present in the cleft epithelium.

In *formalin fixed sections* significant differences could be found with  $\alpha$ -NPr in comparison to  $\alpha$ -NA. The most active hydrolysis of  $\alpha$ -NPr occurred in the pericytic cells (Fig. 20). When this activity was inhibited by F600 (Fig. 21) it became evident that this substrate was also split by a perisinusoidal I<sub>1</sub> activity. This reaction which was much weaker than with  $\alpha$ -NA was present in gonadotropic and thyrotropic cells. This activity was also resistant to NaI and only partially inhibited by DPP. Cu<sup>+</sup> at higher concentration ( $10^{-3}$  M, Fig. 22) was able to extinguish all of this coarse granular activity, but the F600 sensitive pericytic activity was still noticed although markedly decreased. In the presence of F600 and p-CMB simultaneously the pericytic activity was totally inhibited while a clear

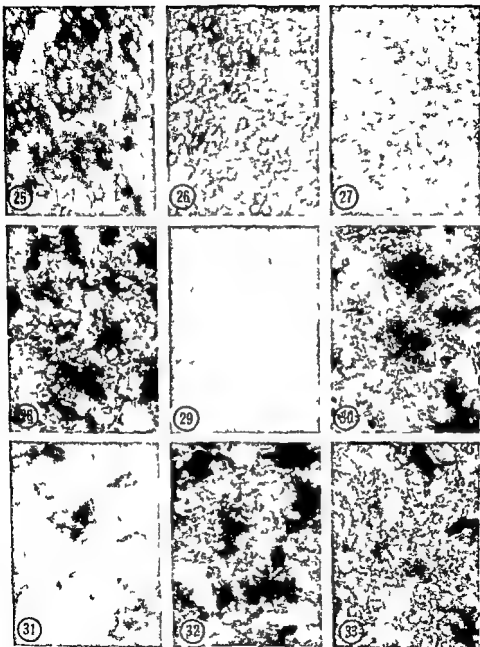


Fig 25  $\alpha$  NB Unfixed section.  $\times 150$   
 Fig 26  $\alpha$  NB Unfixed section. Eserine 10 M  $\times 150$   
 Fig 27  $\alpha$  NB Unfixed section Sodium taurocholate 10 M  $\times 150$   
 Fig 28  $\alpha$  NB Fixed section  $\times 240$   
 Fig 29  $\alpha$  NB Fixed section E600  $10^{-4}$  M  $\times 240$   
 Fig 30  $\alpha$  NB Fixed section Sodium taurocholate 10 M  $\times 240$   
 Fig 31  $\alpha$  NV Fixed section  $\times 240$   
 Fig 32  $\alpha$  NV Fixed section Triton X 100 0.1%  $\times 240$   
 Fig 33  $\alpha$  NV Fixed section Triton X 100 0.1%  $\times 240$



activation of the granular perinuclear activity in gonadotrophic and thyrotrophic cells was observed. The same kind of activity in somatotrophic and ACTH cells was also visualized by this method (Fig 24).

The pericytic activity was clearly activated by the presence of Triton X 100 (0.1 %), but no apparent effect on the L600 resistant parenchymal cell activity was revealed (Fig 23).

A very intense esterase activity was shown in the epithelial cells on both sides of the pituitary cleft with  $\alpha$ NPi. This activity was totally inhibited by L600 NAF as well as by DFP but remained unaffected by eserine. These findings are consistent with those obtained with  $\alpha$ NA as substrate, but this enzyme clearly preferred  $\alpha$ NPi.

**$\alpha$  Naphthyl butyrate** — In fresh frozen sections the hydrolysis of  $\alpha$ NB by the gonadotrophic and thyrotrophic cells as well as the honeycomb like activity was slight in fresh frozen sections but using longer incubation times, a similar reaction was obtained as that with the previous substrates (Fig 25). Very intense activity was always obtained in the pituitary cleft epithelium also in fresh frozen sections. All these activities were sensitive to L600, DFP and NAF but resistant to eserine (Fig 26). The activity in gonadotrophic and thyrotrophic cells was almost totally inhibited by sodium taurocholate (Fig 27).

In formalin fixed sections the most prominent activity shown with  $\alpha$ NB was located in the pericytic cells (Fig 28). This intense hydrolytic activity almost overshadowed the slight activity in gonadotrophic and thyrotrophic cells.  $\alpha$ NB was found to be the preferred substrate for this pericytic activity. This substrate gave also a mild hydrolytic activity in the cleft epithelial cells (Fig 29). All these activities were sensitive to L600 (Fig 29), DFP and NAF. Sodium taurocholate had no apparent effect on the pericytic activity (Fig 30) while Triton X 100 (0.1 %) clearly enhanced this activity.

**$\alpha$  Naphthyl valerate and  $\alpha$  Naphthyl caprylate** — In fresh frozen sections  $\alpha$ NV showed still weaker activity than  $\alpha$ NB and with  $\alpha$ NC no activity was present at all.

In formalin fixed sections  $\alpha$ NV (Fig 31) was hydrolyzed by an activity in the pericytic cells with equal inhibitor characteristics as shown using  $\alpha$ NB as substrate. The cleft epithelium also had a strong activity with  $\alpha$ NV but  $\alpha$ NC gave a negative result. When  $\alpha$ NV (Fig 32) and  $\alpha$ NC (Fig 33) were used with Triton X 100 (0.1 %) the activity was much increased in both the pericytic and the cleft epithelial cells. This effect seems to be due at least partly to a better solubilization of the substrates.

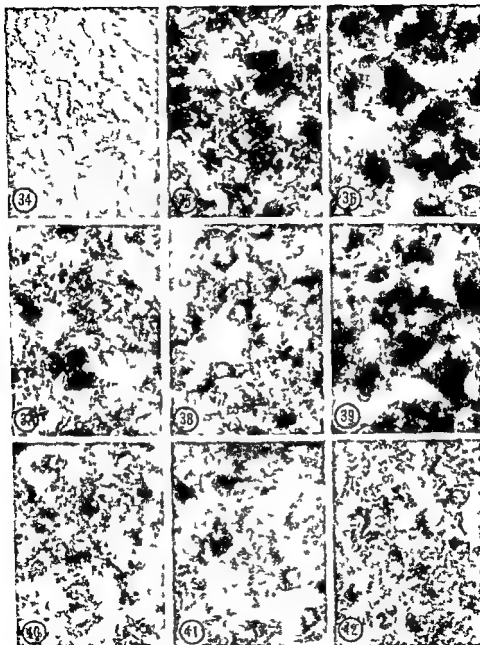


Fig 34 N 19 Unfixed section  $\times 150$

Fig 35 N AS Fixed section  $\times 240$

Fig 36 N AS Fixed section p CMB  $10^{-4}$  M  $\times 240$

Fig 37 N AS Fixed section Eb00  $10^{-4}$  M  $\times 240$

Fig 38 N AS Fixed section. DFP  $10^{-4}$  M  $\times 240$

Fig 39 N AS Fixed section Triton X 100 0.05%  $\times 10$

Fig 40 N AS D Fixed section  $\times 40$

Fig 41 T 11 Fixed section  $\times 240$

Fig 4 T 11 Fixed section F600  $10^{-4}$  M  $\times 240$

*Naphthol AS acetate* — In *fresh frozen sections* no or a very slight activity was observed after a long incubation time (Fig. 34). On the contrary, in *formalin fixed sections* a very intense activity was shown in different cell types (Fig. 35). A strong coarse granular activity was found in gonadotropic and thyrotropic cells and a weaker one in somatotropic and most likely in corticotropic cells. The esterase activity in all these cells was perinuclearly located. Capillary endothelial cells showed also a weak granular activity. No hydrolysis was present in the pericytes or in the cleft epithelial cells in the pituitary gland of the control rat.

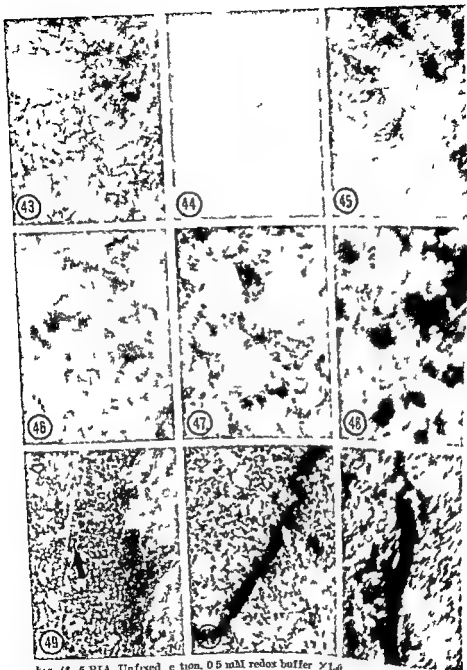
Some activation of the granular reaction was observed in the presence of pCMB (Fig. 36). All the activities obtained were resistant to E600 (Fig. 37). NaF and eserine DFP (Fig. 38) had some inhibitory effect on the granular parenchymal cell activity and almost totally inhibited the hydrolytic reaction in the capillary endothelial cells.  $\text{Cu}^{++}$  also decreased this latter activity. Triton X 100 at 0.05 % concentration had no apparent effect (Fig. 39), but 0.1 % concentration caused some inhibition.

*Naphthol AS D chloroacetate* — In *fresh frozen sections* no activity was obtained with N AS D in any cells of the anterior pituitary gland.

In *formalin fixed sections* both gonadotropic and thyrotropic cells showed an equal and granular, but weak activity, in their cytoplasm when N AS D was used as substrate (Fig. 40). Weak activity was also present in capillary endothelial cells. These activities were resistant to E600 and NaF.

*$\beta$  Naphthyl laurate* — No activity was ever obtained with  $\beta$  NL either in the presence or absence of any activator including sodium taurocholate. The result was also negative in the presence of Triton X 100 in all concentrations tested.

*Thioacetic acid* — TAA as substrate revealed no activity in *fresh frozen sections*. In *formalin fixed sections* a large number of granular dye deposits were present in different cell types (Fig. 41). The most active cells were again both types of gonadotropic cells as well as the thyrotropic cells but additional activity was present in almost every other parenchymal cell except the chromophobic ones. Thioacetic acid esterase activity was also shown in the epithelial cells of the pituitary cleft (Fig. 51). In these cells the activity was mainly diffuse or finely granular. The coarse granular activity in different parenchymal cells as well as in the capillary endothelial cells was resistant to E600 (Fig. 42) and NaF as well as to eserine, thus the same characteristics were obtained as with N AS as substrate. The esterase activity in the cleft epithelial cells was sensitive to E600 and NaF but resistant to eserine, it may be identical with the enzyme preferring a NB as substrate.



- Fig 43 5 BIA Unfixed section, 0.5 mM redox buffer  $\times 140$   
 Fig 44 5 BIA Unfixed section 0.5 mM redox buffer  $\times 140$   
 Fig 45 5 BIA Unfixed section 0.5 mM redox buffer  $\times 140$   
 Fig 46 5 BIA Fixed section, 0.5 mM redox buffer  $\times 230$   
 Fig 47 5 BIA Fixed section 0.5 mM redox buffer  $\times 230$   
 Fig 48 5 BIA Fixed section 0.5 mM redox buffer pCMB  $\times 230$   
 Fig 49  $\alpha$  NA Unfixed section Black arrow shows the adenohypophysis  $\times 60$   
 Fig 50  $\alpha$  NB Fixed section Open arrow shows the adenohypophysis  $\times 60$   
 Fig 51 TAA Fixed section Open arrow shows the adenohypophysis  $\times 60$

**5 Bromoindoxyl acetate** — Using 5 mM concentration of ferro and ferriyanides no activity could be obtained in *fresh frozen sections* (Fig 44) When the ferro ferriyanide redox buffer concentration was reduced to 0.5 mM, esterase activity was present in almost every cell, but most active were, however, the gonadotropic and thyrotropic cells. In Fig 43 the strongest activity is shown in the TSH cells around the sinusoids. This activity was finely granular and distributed all over the cytoplasm but left the nuclei free. It was inhibited by F600 and NAF, but was unaffected by eserine (Fig 45). It seems likely that these activities correspond to those obtained in fresh frozen sections with  $\alpha$ -NA and  $\alpha$ -NPr as substrate.

In *formalin fixed sections* very intense coarse granular, perinuclear activity was present both in gonadotropic and thyrotropic cells when the original 5 mM concentration of ferro ferriyanide redox buffer was used in the incubation medium (Fig 47). The histochemical reaction totally resembled that obtained with N-AS as substrate. When the redox buffer concentration was lowered to 0.5 mM an additional fine granular activity was present in these cells as well as in a number of other cells. Under these conditions the capillary endothelial cells showed also the same kind of fine granular activity (Fig 46). All these hydrolytic activities were resistant to E600 but the capillary endothelial activity was markedly inhibited by  $\text{Cu}^{++}$  and DFP. No activity was present in the pericytes or in the cleft epithelium. The effect of p-CMB was clearly activating on the coarse granular, perinuclear activity in different cell types (Fig 48).

### *Changes in experimental conditions*

Studies on the esterases in normal rat pituitary gland have shown that the ns Es can be grouped into Es ns L and I 1 ns E activities. The former were present both in fresh frozen and in formalin fixed sections, while the latter were revealed only after fixation. N-AS was the preferred substrate for the E600 resistant activities but  $\alpha$ -NA,  $\alpha$ -NPr, and  $\alpha$ -NB must be used for an appropriate demonstration of the different Es ns Ls. These findings are thus applied in the studies concerning the changes in the different esterases due to various experimental states.

### **Cholinesterases**

The hydrolysis of AcThC and BuThC was so weak that no precise estimations of an increase or decrease in various experimental conditions could be made. This is true in both the activity in polygonal TSH cells as

well as the fine granular activity in capillary endothelial cells which was resistant to esterase. The ns ChE in thyrotropic cells was also present in young rats.

#### E 600 sensitive non specific esterases

The hydrolysis of  $\alpha$  NA as well as  $\alpha$  NPr was greatly enhanced in both types of gonadotropic cells after castration when the activity was followed in fresh frozen sections (compare Fig 52 to Fig 53). As a rule this activity is evenly distributed all over the cytoplasm but a longer time after the castration when signet cells appeared it was evident that the hydrolytic activity was absent in the area of the vacuole. In such cases the enzymic reaction was more coarse granulated and perinuclearly located. The same enzymic activity was also increased during pregnancy, but in this case the most active cells were the centrally located LH cells.

The esterase activity in gonadotropic cells as visualized with  $\alpha$  NA and  $\alpha$  NPr was greatly decreased after the treatment with estrogen. The enzymic activity was nearly homogeneous in intensity and very weak in the greatest part of the pituitary gland. The only exception was the TSH cells which after this treatment were clearly found as a separate group of cells in the central part of the pituitary gland (Fig 54). In conditions when a large number of gonadotropic cells with a very intense esterase activity was present it was sometimes difficult to distinguish TSH cells from these.

After progesterone treatment the esterase activity in the central gonadotropic or LH cells was decreased only to a small degree and remained unaltered in all other cell types. Testosterone treatment on the contrary, caused a marked increase of esterase activity in FSH cells, but simultaneously the LH cells seemed to be almost devoid of this type of hydrolytic activity.

The prominent FSH cells which were located in the periphery of the gland in young rats of about one month of age showed an intense activity with  $\alpha$  NA (Figs 55 and 56) and  $\alpha$  NPr, however, the reaction was nearly absent with  $\alpha$  NB.

Changes in the honeycomb like activity in different cell types was also variable in different conditions. During increased gonadotropic secretion such as after castration this background activity seemed to be weaker. This activity was very intense in the young rat pituitary (Fig 55) where it was mainly found in somatotropic cells. The activity was always near to the cell membrane and the central perinuclear area was weaker in activity (Fig 56).

Thyroxine treatment decreased the esterase activity in TSH cells while the contrary was true after methylthiourea treatment (Fig. 57). The background activity representing mainly the somatotrophic cells was however, much weaker in the latter case. The thyrotrophic cells were also easily separable from the gonadotrophic cells due to the more intense activity in the polygonal cells. No change from the control state however, occurred in the esterase content of the gonadotrophic cells.

During lactation the  $E_{s \text{ ns } L}$  in both gonadotrophic cells was minimal and only some weak activity was present in somatotrophic cells. The lactotrophic cells showed very little or none of this type of activity. The same finding was valid also in other states of increased lactotrophic hormone synthesis or secretion such as after estrogen or reserpine treatment.

Correlation of these  $E_{s \text{ ns } E}$  activities in formalin fixed sections appeared to be impossible for a variety of reasons. The most difficult task was to differentiate them from the  $L_{1 \text{ ns } I}$  present in the same cells, but the  $E_{s \text{ ns } L}$  activities in the parenchymal cells were also markedly inactivated during the formalin fixation.

After castration in both sexes the pericytic activity in the area of the TSH cells was markedly increased and extended to the central area of the pituitary gland (compare Fig. 58 to 59). These cells were surrounding the enlarged gonadotrophic cells but were not continuous like the sinusoidal capillary walls. When castration cells appeared, the cytoplasmic activity near the cellular membrane was totally fused with the much greater pericytic activity present when  $\alpha NA$  or  $\alpha NPr$  were used as substrates. These two activities are not, however, identical, since they differ from each other in substrate specificity. The  $E_{s \text{ ns } E}$  in parenchymal cells is probably identical with that found within the same cells in unfixed section. This activity is also markedly inactivated by formalin and becomes covered by the  $L_{1 \text{ ns } I}$ . The pericytic activity was resistant to formalin fixation and was best shown by  $\alpha NB$ . When the pituitary glands of young rats were fixed and the distribution of the hydrolytic activity against  $\alpha NB$  was tested it was shown that only some isolated pericytic activities were found. They were not situated near the TSH cells which presumably are actively secreting.

No consistent change in the  $E_{s \text{ ns } E}$  activity in any parenchymal cell type was observed either during cortisone treatment or after adrenalectomy.

The strong  $E_{s \text{ ns } E}$  activity towards  $\alpha NPr$  and  $\alpha NB$  and to a lesser degree to  $\alpha NA$  and TAA in the cleft epithelium was best followed in formalin fixed sections. It was found also in young rats but the activity was quite weak. This activity was markedly increased after castration.

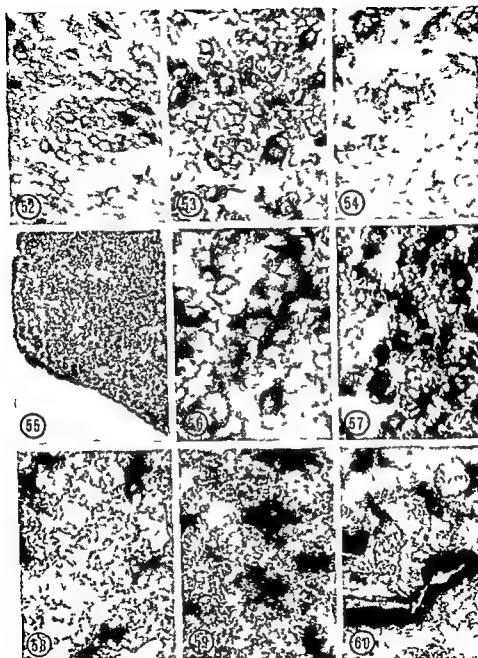


Fig 52  $\alpha$  NA Control female rat Unfixed section  $\times 150$

Fig 53  $\alpha$  NA Female rat castrated 30 days earlier Unfixed section  $\times 150$

Fig 54  $\alpha$  N Female rat after 30 days treatment with estrogen, Unfixed section,  $\times 150$

Fig 55  $\alpha$  N 30 days old male rat Sagittal unfixed section  $\times 60$

Fig 56  $\alpha$  NA Edge area of the Fig 55  $\times 240$

Fig 57  $\alpha$  NA Male rat after 30 days treatment with methylthiourea Unfixed section  $\times 150$

Fig 58  $\alpha$  NB Control male rat Fixed section  $\times 240$

Fig 59  $\alpha$  NB Male rat castrated 30 days earlier Fixed section  $\times 240$

Fig 60  $\alpha$  NB Male rat after 30 days treatment with methylthiourea Fixed section  $\times 240$



(Fig 60) some increase was also found after adrenalectomy. Estrogen and cortisone treatment decreased this activity but progesterone and testosterone were without effect. No apparent change was either shown after thyroxine or methylthiouracil treatment.

#### LEUOCYTES: consistent non specific esterases

The strong  $\text{E r ns E}$  activity obtained with  $\alpha\text{NA}$  (Fig 61) or  $\text{NAS}$  (Fig 64) was much increased in the cytoplasm of both LH and FSH cells after castration (Figs 62 and 65). This augmentation of the activity was apparent 15 days after gonadectomy. Estrogen treatment markedly decreased the granular  $\text{E r ns E}$  in both gonadotropic cell types - the most active cells were then thyrotropic and somatotropic cells (Figs 63 and 66). Testosterone treatment caused no apparent change in the hydrolytic activity of either gonadotropic cell type but the activity in somatotropic cells was somewhat augmented.

In young female rats very intense activity was confined to the centrally situated angular cells - these are presumably actively secreting TSH cells but the peripheral cells having very much  $\text{E r ns E}$  in fresh frozen sections were lacking this  $\text{L r ns E}$  almost totally. Between the active TSH cells other smaller cells with very intense activity were observed. These were mainly growth hormone secreting cells (Fig 67). The degranulated somatotropic cells of the methylthiouracil fed rats showed a moderate coarse granular activity. The thyrotropic cells (Fig 68) showed an increased activity but the  $\text{E r ns F}$  in gonadotropic cells was much decreased during methylthiouracil feeding.

The  $\text{E r ns E}$  was also increased during pregnancy in the central gonadotropic cells and this activity soon decreased after delivery. During the first days after delivery the  $\text{L r ns E}$  in gonadotropic cells decreased rapidly but a similar activity was found in periventricular cells (Fig 69). These cells lost this activity about ten days after lactation. This suggests that the enzymic protein is expelled from the gonadotropic cells and is picked up by the periventricular cells which have phagocytic properties. The lactating rat pituitary has also very much diminished  $\text{E r ns E}$  in the gonadotropic cells and quite weak activity seems to be connected to the LTH cells.

Adrenalectomy had only a slight effect on the  $\text{E r ns I}$  but a moderate increase in the hydrolytic reaction in ACTH cells could be constantly obtained. After cortisone treatment the histochemical esterase reaction was seemingly augmented. This effect was not caused by an increase in esterase activity in any special cell type but was the result of the tighter packing of the active gonadotropic cells in the shrunken pituitary gland.

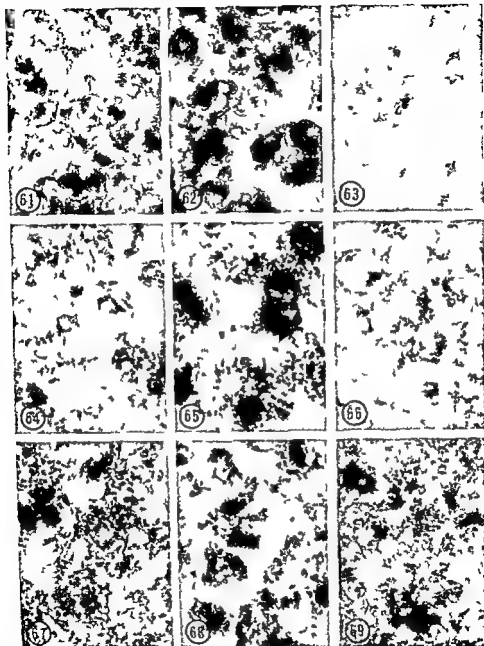


Fig 61 a NA Control female rat Fixed section  $\times 240$

Fig 62 a NA Female rat castrated 60 days earlier Fixed section  $\times 240$

Fig 63 a NA Female rat after 60 days treatment with estrogen Fixed section  $\times 240$

Fig 64 N AS Control female rat Fixed section  $\times 240$

Fig 65 N AS Female rat castrated 60 days earlier Fixed section  $\times 240$

Fig 66 N AS Male rat after 60 days treatment with estrogen Fixed section  $\times 240$

Fig 67 N AS 30 days old female rat Fixed section  $\times 240$

Fig 68 N AS Male rat after 60 days treatment with methylthiourea Fixed section  $\times 240$

Fig 69 N AS Female rat 5 days after delivery Fixed section  $\times 240$

## Discussion

### *Cholinesterases*

The esterase activity in the thymotropic cells had all the characteristics of *ns* ChE because it was inhibited by eserine, iso OMPA and ethopropazine at  $10^{-5}$  M concentration. Since this enzymic activity was found only after formalin fixation, it is probably solubilized during the long incubation time used. The intracellular ChE activity is mainly bound to the ergastoplasmic reticulum as revealed by electron microscopic (Torac and Barnett 1962, Mori et al 1964) and subcellular tissue fractionation studies (Underhay et al 1956, Aldridge and Johnson 1959, Hanson and Toschi 1959, Toschi 1959).

*Ns* ChE as well as AChE are known to hydrolyze also naphthol and indoxyl esters (Pearse 1961). The contribution of the *ns* ChE activity to the high hydrolysis rate of these substrates in the TSH cells in the present investigation seems to be meager, because the effect of eserine was minimal.

The other enzymic activity, which hydrolyzes thiocholine esters is situated perisinusoidally and is not inhibited by eserine or E600. It is neither AChE nor *ns* ChE. Such an enzymic activity has been previously demonstrated in the endothelial lining of the hypothalamic blood vessels of rat but not in hedgehog, ferret, or monkey; thus enzyme does not hydrolyse 5 BIA (Holmes 1961). In the present study this activity could not be clearly correlated with the enzymic activity obtained with other substrates but  $\alpha$  NA and 5 BIA were also hydrolysed by an *E* *ns* *E* activity in the capillary endothelial cells. The actual site of the enzymic activity of this type may also be in the sympathetic ground plexus or secretory motor end plexus (Metuzals 1958) although available studies do not prove the existence of such an innervation in the pars distalis (Green 1961, Harris 1955).

### *E600 sensitive non specific esterases*

The present histochemical study shows that all *E* *ns* *E*s in the parenchymal pituitary cells are optimally demonstrable in fresh frozen sections. This indicates that they are bound to some cellular particles. In studies concerning the distribution of the esterase activity in the subcellular fractions of rat and mouse liver (Novikoff et al 1953, Underhay et al 1956, Carruthers and Braumler 1961, Carruthers et al 1965) and rat brain (Aldridge and Johnson 1959) *L* *s* *ns* *I* activity was mostly present in the

microsomal fraction Studies of Chaveau et al (1962) showed that the site of the esterases was in the membranes present in this fraction

Nonionic detergents, e.g. Triton X 100 are frequently used for the liberation of the lysosomal enzymes (Wattiaux and de Duve 1956) The esterases of the microsomal particles have also been solubilized by anionic detergents (sodium deoxycholate sodium lauryl sulfate sodium cetyl sulfate, sodium dodecyl benzene sulfonate) or by the nonionic detergents (Lubrol W and Tween 80 Carruthers and Baumler 1962) The effect of Triton X 100 on the histochemical esterase reaction in the fresh frozen pituitary section varied somewhat depending on the concentration of the detergent At 0.1 % concentration with 30 min preincubation all hydrolytic activity was lost Using lower concentrations some activity was still present in the cell periphery This difference suggests the presence of two different types of enzymes equal in affecter characteristics but differing somewhat in intracellular location and substrate specificity

The marked increase found in the E<sub>s</sub> ns E activity in gonadotropic cells after castration and in thyrotropic cells after methylthiouracil treatment shows that this enzyme activity is connected to the increased production and/or release of the respective hormones Ribosomes aggregated in the endoplasmic reticulum have been found electron microscopically, indeed, in increasing amounts in gonadotropic cells after castration (Girod et al 1964 Yoshimura and Harumya 1965) as well as in thyrotropic cells after thyroidectomy (Fatouhar and Rinehart 1954 Cardell 1964)

The effect of estrogen and testosterone on the esterolytic activity in the gonadotropic cells is markedly different Estrogen treatment causes almost total depletion of the esterase activity in both LH and FSH cells but testosterone decreases only that in the LH cells and at the same time significantly increases the FSH cell activity These findings are in agreement with the studies concerning the effect of estrogen and testosterone on the synthesis of LH and FSH by the rat pituitary Estrogen has been shown to decrease both the FSH (Paesi et al 1955 van Rees 1961) and LH production (Greep and Chester Jones 1950) while testosterone increases the production of FSH (Hoogstra and Paesi 1957, Paesi et al 1959) and decreases that of LH (Paesi et al 1958)

The site of the other E<sub>s</sub> ns E was mainly in the peripheral part of the somatotropic cells although numerous other cell types showed this type of activity Triton X 100 was able to solubilize this activity too but at higher concentrations This may mean that the site of binding of this enzyme in the molecular cellular particles may be different In increased somatotropic production as could be anticipated in the young rats this

activity was found to be strong, and an apparent contribution of this enzymic activity to the hormone synthesis is thus likely.

The contribution of esterase activity to the release mechanism of the hormone granules must also be taken into account since the intracellular enzymic activity may dissolve the limiting membrane of the hormone granules by their transfer through the cell membrane (Yoshimura and Harumiya 1965). Methylthiourea treatment discharges the granules of the somatotrophic cells but it simultaneously depresses the hormone production. In this state the esterase activity was also found to be decreased. This suggests that also in this cell type the esterase activity is probably connected to the production of the hormone and not to its release. Since the weak E<sub>s</sub> and E<sub>h</sub> in other parenchymal cells showed no consistent changes during experimental conditions their role in the hormone production remains unclear.

The pericytic E<sub>s</sub> and E<sub>h</sub> found only in formalin fixed sections cannot be taken as an enzymic activity derived from the parenchymal cells by excretion because it had clearly differing substrate specificity. The pericytic cells are known to possess phagocytic properties (Cappell 1929) and it is thus to be expected that different hydrolytic enzymes are present. Rinehart and Farquhar (1955) and Farquhar (1961) suggested that these cells probably play an active role in the mechanism of hormonal transfer from the parenchymal cells into the sinusoids. These histiocytic cells may also be active in the phagocytic, digestive, and assimilative function toward the cytoplasmic debris from the parenchymal cells. To this function may belong also the capture of the remnants of the hormone granules and carrier proteins as well as the preparation of some nutritive material for the hormone producing cells. The enzymic activity found in these cells may be active in these processes.

The present study showed the close connection of the esterolytically active pericytic cells with the gonadotropic cells. In the control animal the most active cells were found around the TSH producing cells. After castration and during pregnancy active pericytic cells were also found near the LH cells but were not related to the thyrotropic cells during increased TSH secretion either. Some special function for this esterase activity might thus also be expected and further studies are needed for its elucidation.

The epithelial cells of the residual cleft between anterior and intermediate lobes showed an intense esterase activity preferring about the same substrates as that in the pericytic cells. The role of these cells in pituitary function is still totally unexplained. Some changes were however

noted in these cells in different experimental conditions (Feirer 1956). The marked increase in the esterase activity in these cells after castration and adrenalectomy may probably be connected to the suggested holocrine secretion of these cells in these states. The weak esterase activity in the colloid in the residual cleft may also be released from these cells. It might be suggested that identical activity was found by Jirasek (1963) in certain cells lining the residual follicles in the human fetal pituitary gland.

### *E600 resistant non specific esterases*

Almost all parenchymal cell types show also an  $E_{rnsE}$  activity but most active cells are again thyrotropic and both types of gonadotropic cells and markedly less the somatotropic cells. In contrast to the  $E_{snsE}$  this activity is confined near the nuclei and the enzyme reaction shows coarse granular deposits. In addition some diffuse background activity remains after E600 treatment in the thyrotropic and gonadotropic cells especially when 5 BIA or  $\alpha$  NA were used as substrates. At lower pH's N AS and TAA showed only the granular activity. This suggests that the site of this activity may be the lysosomal particles in which very active  $E_{rnsE}$  has been demonstrated both biochemically (Bernsohn et al 1964; Shibko and Tappel 1964) and electron microscopically (Wachstein and Meisel 1960; Torack and Barnett 1962; Müller 1964) in liver and brain. Whether similar activity present in the capillary endothelial cells is transferred from the parenchymal cells cannot be settled.

Since the granular activity was significantly increased when the sections were preincubated with p CMB it may be related to the C esterase as demonstrated by Bergmann et al (1957) in hog kidney. None of these activities can be cathepsin C because of their insensitivity to SH reagents or diazo salts which totally inhibit the demonstration of the enzyme using simultaneous diazo coupling (Vanha Perttula et al 1965).

The  $E_{rnsE}$  activity seems to be very soluble, although it is suggested at least partly to be localized in the subcellular particles. Lysosomal enzymes are usually quite easily solubilized e.g. by freezing and thawing by nonionic detergents and by hypotonic solutions (de Duve 1959). On the other hand some enzymes located in the microsomal particles are more tightly bound e.g. microsomal aminopeptidase in kidney tissue is rather difficult to liberate (Pfleiderer and Celhiers 1963; Vanha Perttula et al 1966) although esterases seemed to be more easily solubilized as shown in the present study. Differences have also been observed in the site of enzymic location in particles. So acid phosphatase in lysosomes has been

claimed to be inside the particle membrane (de Duve 1959), while L r ns E is perhaps situated on the surface of the particle (Holt 1963, Shibko and Tappel 1964) from which the enzyme can be solubilized although acid phosphatase still remains inside the particles. Previous studies on nervous tissue have demonstrated that L r ns Es are very soluble and cannot be visualized in fresh frozen sections (Esila 1963, Haikonen 1964, Kokko 1965, Soderholm 1965).

Also the L r ns F's are clearly connected to the functional state of the rat pituitary gland. This activity was clearly augmented in gonadotropic, thyrotropic, as well as somatotropic cells in states of increased secretory activity. The coarse granular activity is most active in the Golgi zone and especially in the gonadotropic cells on that side of the cell facing the perisinusoidal space. The Golgi apparatus is intimately connected to the release mechanism of secretory cells (Farquhar 1961). The synthesized protein is condensed to a secretory granule in the canaliculi of the Golgi zone and then migrates to the cell membrane with which it fuses. The cell membrane thins out and an opening forms to the exterior and the granule is dissolved into the perivascular space. The intense L r ns E in this area is probably connected to the disintegration of the lipoprotein membranes of these granules or to the production of them since in cellular metabolism the hydrolytic enzymes may be suggested to have also synthesizing properties.

An interesting finding in the present studies was that after delivery this L r ns L was depleted from the gonadotropic cells. The identical activity was then for some time shown in the pericytic cells which are normally devoid of all L r ns E activity. This finding is in agreement with the suggested phagocytic activity of these cells. The enzyme protein is extruded from the parenchymal cell and is then picked up by the histiocytic cell and gradually disintegrated. This activity may correspond to the 5 BIA hydrolyzing activity in the human pituitary pericyte (Pearse 1956).

# FRACTIONATION OF CARBOXYLIC ESTERASES IN THE RAT ADENOHYPOPHYSIS

## LITERATURE REVIEW

### Earlier fractionation studies of esterases

A method often utilized for the fractionation of enzymes in connection with histochemical investigations is electrophoresis. This is usually carried out on paper or agar gel or some material such as starch gel or acrylamid having a molecular sieving effect in addition (Hunter and Markert 1957 Ornstein and Davis 1962). By these methods esterase activity can be shown by the usual histochemical reactions; the counterpart for the deposition of dye in sections is thus obtained. Moreover, the characteristics of a multiple enzyme system can be tested in the gel slabs using various inhibitors and activators; also the effect of fixatives on the enzyme can be tested. Starch gel electrophoresis has been an especially favored method in the esterase studies (Hunter and Markert 1957, Markert and Hunter 1959, Hunter and Burstone 1960, Allen and Hunter 1960, Eranko et al 1961, b 1964, Enla 1963, Harkonen 1964, Allen et al 1965, Kokko 1965, Soderholm 1965).

By electrophoresis only those esterases which are soluble (lyo enzymes) can be fractionated; the firmly bound (desmo enzyme) are not demonstrable in the electrophoretic slab unless they are first solubilized by freezing and thawing or other procedures. It has thus been found that the primarily soluble esterases are not demonstrable in fresh tissue sections, although they can be visualized in starch gel slabs without any solubilization procedures. Lyo esterases can be separated from the desmo esterases by centrifugation of the tissue homogenates in isotonic solution. Markert and Hunter (1959) showed that in mouse tissues only about 50 per cent of the total esterase activity is primarily electrophoretically mobile.

Enzyme activity is not always demonstrable in a starch gel slab by the usual histochemical procedures possibly because of too weak enzyme activity, low penetration of the substrate to the gel, or inhibition of the enzyme in the presence of the coupling agent used. To detect such enzymes the starch gel slab can be cut transversely and the enzymic activity tested in the eluates obtained after homogenization of such pieces (Vanha Perttula et al 1965).

The size of the crude enzyme samples which can be fractionated by starch gel electrophoresis is limited. Therefore other fractionation techniques must be used to obtain enzyme protein fractions from a larger tissue sample. Cellulose ion exchangers provide a means for the separation of all types of proteins and has also been used for the separation of esterases (Bergmann and Rimoin 1960, Svensmark 1963, Carruthers et al 1965, Vanha Perttula 1964, Vanha Perttula and Hopsu 1965a).



## OWN INVESTIGATIONS

## Material and methods

**Substrates and enzyme assay** — Substrates used in the electrophoretic and quantitative studies were commercial preparations from Sigma Chemical Comp (Ohio, U.S.A.) and are listed in Table VI. This table includes also the final concentration of substrates as well as the incubation times at 37 °C both in the starch gel electrophoresis and in quantitative biochemical studies.

In preparing the stock solutions for staining the electrophoretic slabs 0.02 mmole and for quantitative studies 0.01 mmole of the  $\alpha$ -naphthol substrates were dissolved in 3 ml of methanol; the respective amount of  $\beta$ -naphthol substrates were dissolved in 5 ml of methanol and then diluted with water up to 10 ml.

The incubation medium for the starch gel slabs included

Tris HCl buffer 0.1 M pH 7.0	100 ml
Substrate stock solution	50 ml
Fast Garnet GBC solution (4 mg/ml)	30 ml
Distilled water or inhibitor solution	20 ml

The detection of 5-bromindoxyl acetate hydrolysis in starch gel was made according to the same method as used in histochemical studies with only 0.5 mM concentration of ferro- and ferricyanides.

For the visualization of ChEs in starch gel slabs the Gomori's thiocholine method was applied. After the incubation the slices were rinsed in several changes of hot saturated sodium sulphate solution and immersed for one minute in 1% ammonium sulphide solution saturated with copper sulphide.

**Table VI** Substrates used in starch gel electrophoretic as well as in quantitative enzymic studies with the final substrate concentration and incubation times at 37 °C indicated.

Substrate	Electrophoretic studies		Quantitative enzyme assay	
	Final substrate concentration (mM)	Incubation time (min)	Final substrate concentration	Incubation time
$\alpha$ -naphthyl acetate	0.5	20	0.25 mM	15 min
$\alpha$ -naphthyl propionate	0.5	20	0.25 mM	15 min
$\alpha$ -naphthyl butyrate	0.5	20	0.25 mM	15 min
$\alpha$ -naphthyl valerate	0.5	20	0.25 mM	15 min
$\alpha$ -naphthyl caprylate	0.5	60	0.25 mM	15 min
$\beta$ -naphthyl laurate	0.5	120	0.25 mM	60 min
$\beta$ -naphthyl myristate	—	—	0.25 mM	120 min
$\beta$ -naphthyl palmitate	—	—	0.25 mM	120 min
Naphthol AS acetate	0.5	20	0.25 mM	15 min
Naphthol AS D chloroacetate	0.5	60	—	—
Bromindoxyl acetate	0.5	60	0.25 mM	60 min
Acetylthiocholine	1.0	12 h	0.5 mM	60 min
Butyrylthiocholine	1.0	12 h	0.5 mM	60 min

The quantitative demonstration of the enzymes was performed in the following solution

Tris HCl buffer 0.1 M pH 7.0	10 ml
Substrate stock solution	0.5 ml
Enzyme solution	0.5 ml

It was established that the reaction was linear with respect to time and enzyme concentration in the experimental conditions used

After incubation the liberated naphthol in the quantitative studies was coupled with 0.3 ml of aqueous Fast Garnet GBC (Edward Gurr Ltd London England 1 mg/ml) in which 10% Tween 20 was added to disperse the dye. After two minutes, 0.7 ml of 10 M acetate buffer of pH 4.2 was added. With  $\beta$  naphthyl esters no Tween was added and the dye was extracted with 3.0 ml of ethyl acetate and centrifuged for 10 min at 3000  $\times$ . The absorbancies were read with a Beckman DB spectrophotometer at 540  $m\mu$  for  $\alpha$  naphthol and  $\beta$  naphthol and at 545  $m\mu$  for naphthol AS derivatives. Results are given in  $m\mu$ mole of naphthol liberated in time unit. The calibration curves were made from the dilution series of  $\alpha$  naphthol  $\beta$  naphthol and naphthol AS (Edward Gurr Ltd London England) initially dissolved in methanol (Vanha Pirttula and Hopau 1965a).

The hydrolysis of 5 bromoindoxyl acetate was measured by reading the intensity of the blue indigo color spectrophotometrically at 625  $m\mu$ . Appropriate standards were made from aqueous solutions of indigo blue (Edward Gurr Ltd, London England).

The hydrolysis of acetyl and butyrylthiocholine was determined by a modified method of Ellman et al (1961). The incubation solution was as follows:

Phosphate buffer 0.1 M pH 8.0	20 ml
Substrate stock solution (6 mM) in water	0.25 ml
Dithionitrobenzoic acid (Aldrich Chem Comp Wis USA 0.36 mM in 0.1 M phosphate buffer pH 7.0, in which 6 mg/ 10 ml NaHCO <sub>3</sub> was added	0.25 ml
Enzyme solution	0.5 ml

After incubation the yellow color was measured spectrophotometrically at 412  $m\mu$ . The results are given as changes in absorbancies in time unit.

The effectors used in the present study were the same as previously given in Table III. The modifier agent was included in the incubation medium in the appropriate concentration and half an hour's preincubation at 37 C was performed before the addition of the substrate. In addition the effect of formalin and Triton X 100 on the esterase activities in starch gel slabs was tested at the final concentration of 0.05% and 0.1% respectively.

*Fractionation of pituitary esterases into bound and soluble fractions* — 500 mg of fresh rat pituitary tissue was homogenized in 50 ml of 0.25 M sucrose solution at +4 C. Ten ml of this solution was diluted to 200 ml with water and used as the total homogenate stock solution. The remaining 40 ml of the homogenate was adjusted to pH 5.0 with 0.1 M HCl after standing for one hour the sample was centrifuged for 60 min at 15,000 rpm in an International high speed refrigerated centrifuge model HR 1 (International Equipment Co Mass USA). The supernatant fraction (pH 5 supernatant) was neutralized and diluted with water up to 400 ml. The sediment was suspended in 40 ml of 0.25 M sucrose and half of this fraction was diluted to 200 ml with water.

(pH 5 sediment) To the other half, 0.05 % Triton X 100 was added. After standing 60 min, this fraction was centrifuged as reported earlier. Both the supernatant and sediment fractions were diluted to 100 ml (Triton supernatant and Triton sediment fractions) with water after centrifugation.

**Starch gel electrophoresis** — The technique used in the starch gel electrophoresis was in principle the same as that of Markert and Hunter (1959). For this study the anterior pituitary tissue was frozen at  $-40^{\circ}\text{C}$ . A sample of 100 mg of tissue was homogenized in 0.3 ml of distilled water with a teflon pestle glass homogenizer (Thomas Philadelphia, U.S.A.). The homogenate was frozen and thawed five times and centrifuged for 10 min at  $20,000 \times g$ . One tenth ml of the supernatant solution was pipetted on to stripes of Whatman No. 1 filter paper ( $8 \times 0.6$  cm). A piece of such paper was inserted into a slit made with a razor blade in the gel. In preparing the starch gel 36 % of hydrolyzed starch (Connaught, Toronto, Canada) was mixed and boiled with 300 ml of 0.076 M Tris HCl buffer, pH 8.65, and poured into a plastic tray (dimensions  $25 \times 8 \times 0.6$  cm). A transverse slit was cut 5 cm from the cathodal end of the tray. The bridge buffer used in both sides was 0.3 M boric acid NaOH buffer, pH 8.0. Eight filter paper sheets connected the buffers to both ends of the gel tray.

Electrophoresis was carried out for 4 hours with a voltage gradient of 10 volts/cm using an LKB electrophoresis power supply (LKB Produkter, Stockholm, Sweden). During the electrophoresis the gel was covered with a plastic foil to avoid evaporation and cold water was running beneath the gel to keep the temperature near  $+4^{\circ}\text{C}$ . During the electrophoresis the anodal front migrated 15 cm. After electrophoresis the starch plate was sliced longitudinally into ten slabs, each 0.8 cm broad. After histochemical reaction the slabs were rinsed in water and stored in glycerol.

**DEAE cellulose column chromatography** — The techniques used for esterase chromatography have been reported in a separate communication (Vanha Perttula and Hopsu 1965a).

## Results

### *Studies with tissue homogenate*

In the present studies of a homogenate and its fractions after centrifugation and treatment with Triton X 100 the hydrolytic activities were tested in 0.1 M Tris HCl buffer of pH 7.0 regardless of the preferred pH area for the individual substrates earlier described by Vanha Perttula and Hopsu (1965a). The results are presented in Table VII. It was found that during the fractionation procedures the hydrolytic activities increased differently with respect to different substrates. The hydrolytic activities of the Triton fractions and the pH 5 supernatant were pooled and the percentage increase was calculated for the different substrates as follows:  $\alpha$  NA 75.5 %,  $\alpha$  NB 122.6 %, NAS 13.6 %, 5 BIA 19.4 %, and  $\beta$  NP 42.6 %.

The esterases hydrolyzing  $\alpha$  NA,  $\alpha$  NB, and  $\beta$  NP seem to be particle bound to a great extent whereas NAS and 5 BIA are hydrolyzed better

Table VII Hydrolysis of different ester substrates by the pituitary homogenate, the soluble and particle fractions and by the fractions after the extraction of the bound esterases by Triton X 100 Also shown are the percentage changes in the hydrolytic rates after some modifier substances<sup>a</sup>

Substrate $\alpha$ naphthyl acetate								
Fraction	Hydrolysis rate ( $\mu$ mole/min)	EG00 10 $\mu$ M	E crine 10 $\mu$ M	NaF 1 mM	P CMB 0.1 mM	TC 1 mM	Perazine 1 mM	Cu 0.1 mM
Total homogenate	2589	-84	0	-56	0	-51	-22	-20
pH 5 supernatant	1726	-73	0	-67	0	-37	-30	-17
pH 5 sediment	2953	-97	-3	-64	-3	-46	-17	-24
Triton supernatant	1151	-89	-6	-54	-8	-77	-24	-40
Triton sediment	1663	-86	-3	-72	-6	-34	-27	-23
Substrate $\alpha$ naphthyl butyrate								
Total homogenate	4257	-91	0	-91	0	-19	-24	-10
pH 5 supernatant	3910	-99	0	-90	0	-28	-18	0
pH 5 sediment	4200	-99	0	-99	-8	-14	-14	0
Triton supernatant	2158	-99	-3	-98	-19	-40	-23	-6
Triton sediment	3349	-93	-1	-90	0	-7	-18	0
Substrate naphthol AS acetate								
Total homogenate	0921	-6	0	-2	+31	-12	0	-47
pH 5 supernatant	0652	-2	0	0	+40	-2	0	-13
pH 5 sediment	0342	-7	-2	-7	+14	-14	0	-29
Triton supernatant	0223	-10	0	0	+10	-39	0	-27
Triton sediment	0171	-5	0	-2	-16	0	0	-24

Table 11 (continued)

Substrate 5-hydroxytryptophan								
Fraction	Hydrolysis rate ( $\mu\text{mole}/10\text{ min}$ )	$1600$ $10\text{ } \mu\text{M}$	E. coli $10\text{ } \mu\text{M}$	NaF $1\text{ mM}$	pCMB $0.1\text{ mM}$	TO $1\text{ mM}$	Pernase $1\text{ mM}$	Cu $0.1\text{ mM}$
Total homogenate	3380	0	0	-39	+16	-36	—	-93
pH 7.5 supernatant	1920	0	0	—	+16	-27	—	-93
pH 7.5 sediment	1570	-3	-5	-53	-6	-34	—	-79
Triton supernatant	1403	-17	-17	-73	-15	-30	—	-68
Triton sediment	6722	-5	-13	-50	0	-26	—	-84
Substrate $\beta$ -naphthyl palmitate								
Total homogenate	0.598	-15	0	0	-34	-86	-79	-78
pH 7.5 supernatant	0.136	-88	0	-12	-83	-32	-30	-74
pH 7.5 sediment	0.347	-43	0	-14	-93	-95	-99	-90
Triton supernatant	0.239	-25	0	-15	-77	-92	-99	-94
Triton sediment	0.478	-25	0	-5	-19	-84	-99	-98

by the soluble fraction Triton X 100 solubilized about 40 % of the enzymes hydrolyzing  $\alpha$  NA and  $\alpha$  NB 33 % of the enzymes hydrolyzing  $\beta$  NP, and about 60 % of the enzymes hydrolyzing N AS and 5 BIA from the pH 5 sediment fraction

The enzymes hydrolyzing  $\alpha$  NA and  $\alpha$  NB and also those hydrolyzing  $\beta$  NP are readily inhibited by L600 the two former also are markedly inhibited by NaF Only the enzymes liberated from the particles were sensitive to L600 when N AS and 5 BIA were used as substrates The contribution of ChLs to the hydrolysis of these substrates seems to be slight p CMB seems to inhibit markedly the hydrolysis of  $\beta$  NP, but has a significant activating effect on the hydrolysis of N AS and 5 BIA while the hydrolysis of  $\alpha$  NA and  $\alpha$  NB is only slightly modified Sodium taurocholate (TC) as well as perazine inhibited moderately the hydrolysis of  $\alpha$  NA and  $\alpha$  NB but they markedly inhibited that of  $\beta$  NP The hydrolysis of N AS was not modified by perazine (turbidity prevented the measurement of the hydrolysis of 5 BIA)

$\text{Cu}^{++}$  almost totally inhibited the hydrolysis of 5 BIA and diminished also markedly the hydrolysis of  $\beta$  NP and N AS  $\alpha$  NB was almost unaffected The discrepancy between the reaction of N AS and 5 BIA to  $\text{Cu}^{++}$  inhibition was somewhat surprising since these substrates had otherwise analogous reactions It was observed, however, that  $\text{Cu}^{+}$  in biochemical enzyme studies can inhibit the blue indigo color formation and this can be prevented by adding ferri and ferriyanide in 0.5 mM concentration to the incubation medium as is done in the histochemical method

### *Starch gel electrophoresis*

#### Histochemical staining of the starch gel slices

*Thiocholine esters* — By the present method it was not possible to localize the activities which hydrolyze AcThC and BuThC even when using the long incubation times as in the histochemical method

*$\alpha$  Naphthyl acetate* — This substrate produced the largest number of bands (Fig 70 Table VIII) Altogether 11 positive bands were identified which are designed 1—11 and counted from the anodal end to the origin (Webb 1965) No bands were obtained on the cathodal side with any of the substrates used under these electrophoretic conditions The most active bands were 2 3 4 and 7—11, of which bands 7—11 were near each other Bands 10 and 11 remained after incubation with L600 (Fig 70) Other L 600 resistant activity bands were 2 and 3 The others

were clearly inhibited also by  $\text{NaF}$  DFP inhibited markedly all enzymic activities  $\text{Cu}^{++}$  inhibits moderately activity zones 10 and 11 and totally band 11 Formalin treatment totally inhibits bands 7—9 which are also somewhat inhibited by Triton X 100 in 0.1 % concentration Bands 10 and 11 are somewhat activated by p CMB and band 4 by Triton X 100

*$\alpha$  Naphthyl propionate* — The most apparent difference between  $\alpha$  NP1 and  $\alpha$  NA was the weakness of the bands 10 and 11 (Fig 70) which after E600 treatment were only slightly visible Without E600 this activity was for the most part covered by very intense activity of bands 7—9 Also the D rns E bands 2 and 3 were clearly decreased in activity On the other hand, activity bands 5 and 6 as well as 1 were clearly more active However, the formalin sensitive bands 7—9 (Fig 70) and band 4 were the most active bands obtained

*$\alpha$  Naphthyl butyrate* — Bands 3 10 and 11 were absent with  $\alpha$  NB (Fig 70) Bands 1 5 and 6 were increased in activity but on the whole bands number 4 8 and 9 were most active Band 2 and 7 were quite weak All these activity bands except band 2 were totally inhibited by E600 (Fig 70) and  $\text{NaF}$  Bands 5 and 6 were best visualized with this substrate It was found that when the homogenate was made only of the lateral parts of the pituitary gland this activity could not be found Bands 5 and 6 were most active when starch gel was carried out with a homogenate of the cleft epithelium area of the pituitary gland (Fig 70) or with a homogenate of the intermediate lobe This suggests that these two activity zones were derived from the cleft epithelial activity The characteristics of this activity are clearly reminiscent of those in the histochemical study

*$\alpha$  Naphthyl isobutyrate  $\alpha$  naphthyl caprylate and  $\beta$  naphthyl laurate* — With  $\alpha$  NI (Fig 70) the same activity bands were obtained as with  $\alpha$  NB although they were much weaker The decrease was most apparent in bands 7—9 however band 7 was never seen with certainty With  $\alpha$  NC only bands 1 and 4 appeared (Fig 70) Both these activities were inhibited by taurocholate With  $\beta$  NL no activity could be produced by the usual method or by preincubating the starch gel slab without Fast Garnet GBC and making a postcoupling reaction

*Naphthol AS acetate and naphthol AS D chloroacetate* — Zymograms produced with these substrates showed somewhat different band patterns N AS (Fig 70) was hydrolyzed most strongly by E600 resistant bands 10 and 11 Bands 2 and 3 were also clearly positive The only E600 sensitive activity was band 4 which showed slight activity with this substrate The enzyme activities revealed by N AS D (Fig 70) were much weaker than those of N AS This substrate showed that band 2 was the most active but a slight activity was found also in bands 4 10 and 11

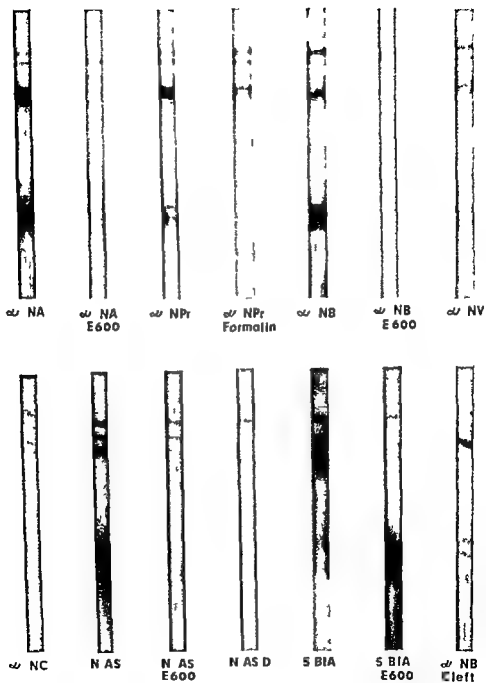


Fig 70 Esterases in zymograms obtained with different substrates and inhibitors. Incubation times for the different substrates are given in Table VI



*5 Bromoindoxyl acetate and thioacetic acid* — Zymograms produced with 5 BIA using a modified indoxyl esterase method in which the ferro-ferricyanide redox buffer was reduced to 10 per cent (0.5 mM) of the original histochemical method gave a positive enzyme reaction in bands 2, 3, 4, 10 and 11 (Fig 70). The relative intensities of these bands varied in relation to those obtained with  $\alpha$ AS. Bands 2 and 4 were clearly more active. Only band 4 was inhibited by F600 (Fig 70). Triton X-100 or formalin had no effect but pCMB had some activating influence on bands 10 and 11. Identical activity bands were obtained using TAA. Pseuine was without effect with all substrates tested in starch gel electrophoresis. This shows the minimal contribution of ChEs in the enzymic composition of rat pituitary gland.

*Substrate preference of the different activity bands (Table VIII)* — Starch gel bands 10 and 11 were always demonstrated by the same substrates at about equal intensity. They may be called *acetylesterases* because they were preferentially revealed by  $\alpha$ AS and well demonstrated also by  $\alpha$ NA, 5 BIA and TAA. Weak activity was found with  $\alpha$ ASD and  $\alpha$ NPr. This enzyme was resistant to F600 but pCMB had a clearly activating effect. These are the characteristics of C-esterase as presented by Bergmann et al (1957) and Bergmann and Rimon (1958, 1960). The intensity of the hydrolysis of TAA can not be correlated because of different conditions in the incubation medium.

Bands 7–9 were best visualized with  $\alpha$ NPr. This was followed with about equal intensity by  $\alpha$ NA and  $\alpha$ NB. Band 7 was however clearly weaker with  $\alpha$ NB than with  $\alpha$ NA. This band was always absent with  $\alpha$ NV but bands 8 and 9 could be clearly shown.  $\alpha$ NC,  $\alpha$ AS, and 5 BIA showed no activity in these bands or the activities may be hidden by bands 10 and 11.

Bands 5 and 6 have equal enzymatic characteristics for substrate specificity and inhibitors. The most representative substrate was  $\alpha$ NB. Both shorter and longer chained naphthol substrates were hydrolyzed with decreasing intensity.

Band 4 represents an  $E_{1/2}$  activity and can be revealed about equally well with  $\alpha$ NA,  $\alpha$ NPr and  $\alpha$ NB. The hydrolytic activity with  $\alpha$ NV and  $\alpha$ NC was clearly smaller but it was, however, the most active band. This band was also shown by  $\alpha$ AS,  $\alpha$ ASD and 5 BIA. This band represents the only  $E_{1/2}$  activity obtained with these substrates.

Band 3 was obtained with all acetate esters and only a very weak activity was found with  $\alpha$ NPr. This enzyme thus represents the same kind of *acetylesterase* activity as enzymes in bands 10 and 11. The relative

substrate specificity between different acetate esters however is different to some extent. The most preferred substrates were  $\alpha$  NA and  $\delta$  BIA, while NAS gave clearly weaker intensity in this band.

Band 2 also hydrolyzed preferentially acetate esters but a clear hydrolytic activity was found also using other ester substrates as long as  $\alpha$  NV. With  $\alpha$  NC the reaction was negative. This band represents the only E<sub>1</sub> or E<sub>2</sub> found with  $\alpha$  NB or longer chained substrates. This enzymic band somewhat preferred NAS in relation to  $\delta$  BIA while the contrary was true in band 4.

Band 1 contained the most mobile enzymic activity. The preferred substrate was  $\alpha$  NB although  $\alpha$  NP<sub>1</sub> seems to be hydrolyzed at about an equal rate.  $\alpha$  NA and  $\alpha$  NV are weaker substrates and with  $\alpha$  NC only minimal activity could be revealed. This enzyme activity was sensitive to U600 and NaF.

*Bound and soluble esterases* — When starch gel electrophoresis was carried out using the supernatant of the pituitary homogenate and correlated with that obtained with whole tissue homogenate it was evident that some esterase bands showed clearly decreased enzymic activity. The most apparent decrease was found in the bands 7—9 which thus seem to represent particle bound esterase activities. All the other activities are apparently more easily solubilized although they may in tissue be bound to some particles. When the sediment fraction was solubilized by repeated freezing and thawing after which electrophoresis was carried out, the most active bands were 7 to 11. This demonstrates that also bands 10 and 11 may be largely particle bound but easily released into solution.

Bands 1—6 are found in the electrophoretic pattern of the supernatant fraction and are thus clearly soluble or free enzymes.

### Studies with starch gel eluates

When starch gel slabs were cut transversely and the activity hydrolyzing AcThC and BuThC determined from the eluate two stronger hydrolytic areas were obtained with both of the substrates (Fig 71). The former had its activity maximum in sections 5—6 which was not coincident with any of the previously reported activity bands in starch gel slabs. This activity was resistant to eserine and U600. The second activity had its maximum in sections 11—12 thus corresponding to the area of the esterase bands 7—11. This activity was totally inhibited with eserine while the bands corresponding these fractions were resistant to it. Some slight inconsistent hydrolysis of the thiocholine esters was observed also in sections further toward the anodal side.

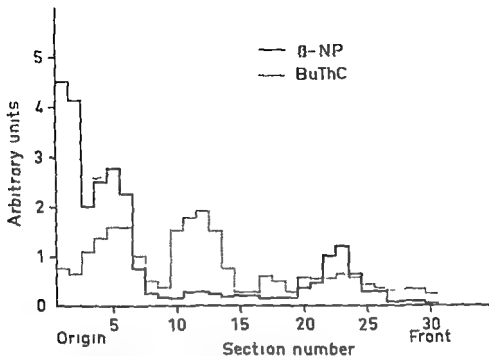


Fig 71 Distribution of the hydrolytic activities toward  $\beta$  NP and BuThC after electrophoretic separation of the pituitary homogenate in starch gel.

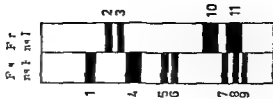
The hydrolysis of  $\beta$  NL,  $\beta$  NM and  $\beta$  NP was also tested with the eluates (Fig 71). It was found that all these substrates were markedly hydrolyzed by the very first sections which usually showed much weaker activity against the short chained fatty acid esters. This activity was markedly inhibited by 1600 taurocholate and perazine. The second activity area was coincident with the first activity against thiocholine esters. This activity was not inhibited by 1600 but some inhibition was obtained with taurocholate and perazine. A third stronger activity was observed in sections 22–23 which corresponded to the area of the 4th activity band in starch gel. This activity was most prominent with  $\beta$  NL of the long chained ester substrates.

#### Correlation of esterases in sections and zymograms (Table VIII)

The strong F<sub>1</sub> and F<sub>2</sub> activities in gonadotropic, thyrotropic and somatotrophic cells most likely correspond to the activity bands 10 and 11 in zymograms. These two bands may represent different cellular origins, which however cannot be demonstrated by substrate specificity or in

Fig. 111 Schematic xymogram illustrating the results obtained by starch gel electrophoresis with different substrates and offering the suggestion of a probable correlation with the site of activity in histochemical studies

Band	$\alpha$ NA	$\alpha$ NPr	$\alpha$ NB	$\alpha$ NV	$\alpha$ NC	NAS	NASD	5BLA	TAL	Site of activity in pituitary tissue
1	++	++	++	++	++	+	+	+	+	pericytic cells (1)
2	++	++	+	+	+	+	+	+	+	diffuse activity in fixed T <sub>4</sub> H & I H cells
3	+	+	+	+	+	+	+	+	+	capillary endothelium
4	+	+	+	+	+	+	+	+	+	pericytic cells
5	+	+	+	+	+	+	+	+	+	cleft epithelium
6	+	+	+	+	+	+	+	+	+	activity in different cell types in unfixed sections
7	+	+	+	+	+	+	+	+	+	,
8	+	+	+	+	+	+	+	+	+	granular activity in fixed T <sub>4</sub> H I H & T <sub>4</sub> H cells
9	+	+	+	+	+	+	+	+	+	,



hibitor studies, such studies do not differentiate these enzyme bands. Both these bands were clearly stronger after castration which has the most effective action on the granulosa cells in fixed sections.

Bands 7—9 were almost totally inhibited by formalin which in histochemical reactions was found to inhibit the  $\Gamma$ 's and  $\Gamma$ 's in unfixed sections. These three bands may thus be derived from various cell types which have the same type of enzymic activity tested in fresh sections. Band 7 was weaker than bands 8 and 9 when  $\alpha$  NB was used as substrate and was found no more with  $\alpha$  NV. This may suggest somewhat different characteristics of this enzyme in different cellular locations. After castration bands 8 and 9 were clearly much increased; these bands may represent the activity found in LH and FSH gonadotropic cells although the identical activity in the thyrotropic cells may contribute. The 7th band may then represent the activity in the somatotrophic cells. Triton X 100 (0.05%) gave a slight inhibition of bands 7—9 when  $\alpha$  NP1 was used as substrate.

Bands 5 and 6 were not present in starch gel when only the lateral part of the pituitary gland was taken for the homogenate but they were very strong in preparations in which also the cleft epithelial tissue was included. A strong enzyme activity at the same level was obtained when starch gel was carried out using intermediate lobe homogenate. The substrate specificity of these bands clearly corresponds to those obtained in histochemical studies on cleft epithelium.

Band 4 had the largest substrate spectrum and was I 600 sensitive. It was not inhibited by formalin treatment; on the contrary, it was somewhat activated by Triton X 100 which was always more marked when the chain length of the substrate increased. The same effect was found in the peroxidic enzyme activity. This enzyme activity was also very much increased after castration but no increase was found after methylthiourea treatment; this effect was found in histochemical studies too.

Bands 2 and 3 are probably different enzymes on the basis of the difference in substrate specificity. Band 3 was best revealed by  $\alpha$  NA and 5 BIA; these are the substrates which show the greatest diffuse activity which was localized in capillary endothelial cells in formalin fixed sections. The diffuse  $\Gamma$ 's and  $\Gamma$ 's in the FSH and LH cells was visualized best by acetate esters but some weak activity can be shown with other substrates too and may correspond to band 2 activity. Such an activity was also obtained with  $\alpha$  ASD.

Band 1 was very sensitive to F600 but was unaffected by formalin. The corresponding histological site is at present unclear. This band was not clearly increased in activity in relation to other activities after castration.

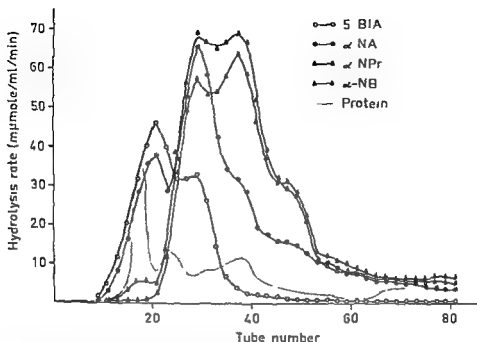


Fig 7\* Separation of the enzymic activities hydrolyzing different esters & substrates on a DEAE cellulose chromatography fractionation at pH 8.2 Incubation times for different substrates are given in Table VI

#### DEAE cellulose column chromatography

This part of the study has been the subject for a separate communication (Vanha Pertturi and Hopsu 1965a), where the esterase fractions at pH 7.0 are described in detail. Therefore only those results will be described here which were obtained by chromatography at pH 8.2 and which differ essentially from those at lower pH.

The distribution of the hydrolysis of ester substrates chromatographed at pH 8.2 is given in Figs 7.2 and 7.3. As a rule poorer separation of the different activity peaks was obtained in comparison to the chromatography at pH 7.0. However, at pH 8.2 the first  $E_{sNsL}$  peak could be divided into two parts with different substrate specificities.  $\alpha$  NA was best hydrolyzed in the former part of the peak (Fig 7.2 tube 29) and showed clearly weak activity in the later tubes where however marked hydrolysis of  $\alpha$  NPr,  $\alpha$  NB,  $\alpha$  NV and  $\alpha$  NC (Fig 7.3 tube 39) occurred. No differences in the inhibitor characteristics in the different parts of the peak could be detected.

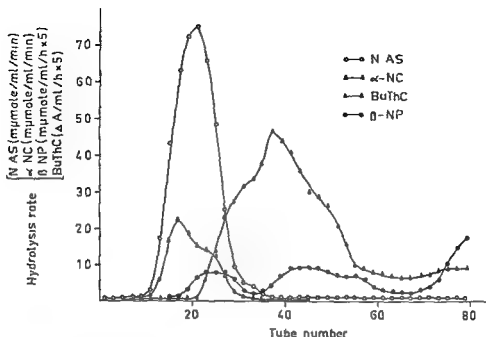


Fig 73 Separation of the enzymic activities hydrolyzing N AS  $\alpha$ -NC BuThC and  $\beta$ -NP on a DEAE cellulose chromatography fractionation at pH 8.2 Incubation times for the substrates are given in Table VI

The fourth activity peak was most active in the hydrolysis of long chained fatty acid esters and it was not totally eluted out of the column by the 0.35 M NaCl concentration at pH 8.2 (Fig 73 tube 80). The hydrolytic activities against thiocholine esters were not favorably separated from the  $\beta$ -NP peak in the pH 8.2 chromatography.

### Discussion

The present fractionation study of rat pituitary homogenate by chromatography and electrophoresis shows the presence of a variety of esterolytic activities. Most of the esterase fractions differ from each other in respect to substrate specificity, modifier characteristics and pH optima, this makes it possible to estimate their activity separately even in a non fractionated pituitary. However, there are different variants of both E<sub>s</sub> and E<sub>i</sub> and E<sub>i</sub> and E<sub>i</sub> activities which cannot be separately analysed.

The four E<sub>s</sub> and E<sub>i</sub> bands in starch gel electrophoresis were not separable in chromatographic fractionation. This activity represents a typical activity

esterase activity (Hofstee 1960) with decreasing substrate specificity order of N AS,  $\beta$  BIA,  $\alpha$  NA and  $\alpha$  NPr. The E 1 ns E runs in the electrophoretic fractions labelled by numbers 10 and 11 in this study but a minor portion of it forms also the fractions 2 and 3. The latter differ somewhat in their substrate preference from the activities in bands 10 and 11. These enzyme activities may represent either isozymes or may be derived from different cell types producing nearly identical enzymes in other characteristics except for a slight difference in electrophoretic mobility. In histochemical studies this type of activity was present in formalin fixed sections in different cells, mainly however in gonadotropic, thyrotropic, and somatotropic cells.

The term isozymes was primarily introduced to describe an electrophoretically separable group of enzymes with otherwise identical enzymatic characteristics (Markert and Moller 1959). This enzymic heterogeneity may be due to the occurrence of a truly different species of native proteins or of precursors or degradation products or of proteins modified *in vivo* or in the preparatory procedure. In human serum and different tissues a group of electrophoretically and chromatographically separable ChEs have been demonstrated which differ from each other with respect to their sialic acid content without any difference in other molecular properties (Svensmark 1965). In a recent study Cann (1966) has demonstrated that a purified protein molecule (bovine serum albumin) in electrophoresis interacts reversibly with borate buffers resulting in multiple protein zones which however do not indicate the presence of altered protein structure. In the present study the electrode buffers also contained borate and it is probable that e.g. bands 10 and 11 may represent a single enzymic species which during the electrophoretic separation has reacted reversibly with the buffer ions.

The possible identity of E 1 ns E with other known enzymes has been much debated. Originally it was suggested on the basis of histochemical studies of Pepler and Pearse (1957) and Hess and Pearse (1958) that the enzymic activity hydrolyzing  $\beta$  BIA may be cathepsin C because this proteolytic enzyme had been shown to exhibit esterolytic activity against substrates like glycyl phenylalanyl glycyl tyrosyl and prolyl phenylalanyl ethyl esters (Wiggans et al 1954). Studies with preparation of hog kidney (Vanha Peritula et al 1965) however showed that cathepsin C is separate from E 1 ns E. Observations using a new cathepsin C substrate glycyl phenylalanyl  $\beta$  naphthylamide have demonstrated that rat pituitary tissue has minimal or no hydrolytic activity toward this substrate at the pH range of cathepsin C although it was used in the presence of cysteine and



EDTA which are known to activate this enzyme (Vanha Perttula, unpublished observations)

It has also been suggested (Holt 1963) that another proteolytic enzyme cathepsin D may be involved. This enzyme hydrolyzed hemoglobin at acid pH (Press et al 1960). When the hydrolysis of hemoglobin was tested in fractions of DEAF cellulose chromatography at pH 3.8, no significant hydrolytic activity coincided with the P<sub>1</sub> and E peak (Vanha Perttula and Hopsu 1965a). The esterase peaks were neither coincident with the activities hydrolyzing leucyl  $\beta$ -naphthylamide (Vanha Perttula and Hopsu 1965b). In pituitary homogenates the hydrolysis of N<sub>2</sub>AS and 5 BIA is somewhat activated by low concentrations of organic mercurials e.g. pCMB but after further purification this activation is lost. These characteristics as well as the pH optimum for the hydrolysis are the same as those presented for a new esterase type called C-esterase by Bergmann et al (1957) and Bergmann and Rimon (1958, 1960).

In starch gel electrophoresis 7 different E<sub>1</sub> and E<sub>2</sub> bands were obtained. They could be partially correlated with the histochemical localization on the basis of substrate specificity and differences in experimental conditions. Three enzymes which migrated the shortest distance in the electrophoresis were found to be identical with the desmo enzymes which were responsible for the histochemical reaction in unfixed section.

Although there are different enzyme bands in starch gel electrophoresis which are similar in isoelectric studies, this does not mean that the enzymes can be termed isozymes. As in the present investigation there is reason to suggest that enzymes of similar modifier characteristics have different cellular locations and slightly different substrate preferences. With suitable modifier substances these may be divided into clearly different subgroups. E<sub>1</sub> and E<sub>2</sub> bands 5 and 6 as well as 7—9 have nearly identical characteristics with respect to substrate specificity. The former correspond clearly to the E<sub>1</sub> and E<sub>2</sub> activity in the cleft epithelial cells and the three latter bands which were sensitive to formalin treatment are the counterpart for the fine granular cytoplasmic activity in gonadotrophic, thyrotrophic, and somatotrophic cells. The question arises whether they are really derived from the different cell types or represent only an enzymic heterogeneity which is produced by the borate buffer during the electrophoresis (Cann 1966). In any case the four activity groups (1—4, 5—6 as well as 7—9) can be taken as separate E<sub>1</sub> and E<sub>2</sub> which also have clearly different locations in the rat anterior pituitary cells and after fractionation by DEAF cellulose chromatography.

The first P<sub>1</sub> and E<sub>1</sub> peak in pH 8.2 chromatography preferred  $\alpha$ -NPr as

substrate, this was also the best substrate for the bands 7—9. The second half of this peak which was more evident in the chromatography at pH 8.2 than at pH 7.0 (Vanha Perttula and Hopsu 1965a), hydrolyzed  $\alpha$ -NB and  $\alpha$ -NV about equally as well as  $\alpha$ -NPr while  $\alpha$ -NA seems to be much inferior as a substrate. This peak most probably corresponds to activity band 4 in starch gel electrophoresis and the pericytic activity in the histochemical reaction. The esterolytic activity in the cleft epithelial cells preferred the same substrates as the pericytic activity. In chromatography at pH 7.0 a small separate F's ns L peak was found (Pial III Vanha Perttula and Hopsu 1965a), in the pH 8.2 chromatography it is only partially separate from the earlier peak. This peak has the same substrate specificity in addition to identical reflector characteristics as the electrophoretic bands 5 and 6.

Band 1 differed from the other F's ns L's in substrate specificity.  $\alpha$ -NPr and  $\alpha$ -NB were the preferred substrates and no hydrolysis of  $\alpha$ -AS,  $\alpha$ -AS-D and 5-BIA occurred. This activity was weaker than the other F's ns L bands and its counterpart in the chromatography may hide behind the other peaks. Its histochemical localization is also uncertain. It might be a second enzyme species in the pericytic cells or correspond to the weak parenchymal E's ns F activity in fixed gonadotropic cells.

The demonstration of AChE and nAChE activities in the histochemical work using AcThC and BuThC as substrates has been the preferred method using Gomori's modification (1952) of Koelle's method (1951). Indoxyl (Adams 1949, Holt 1952) and naphthol esters (Pepler and Pearse 1957, Pearse 1961) have also been used for the demonstration of ChEs and differentiated from the nAChEs by specific inhibitors. The nAChE activity in the rat pituitary thiotropic cells was so weak that its contribution to the hydrolysis of these latter substrates could not be revealed. In quantitative homogenate studies with  $\alpha$ -NA as substrate the inhibition of the hydrolysis was minimal by eserine. In starch gel electrophoresis no eserine sensitive band could be found. Quantitative studies using the method of Ellman et al. (1961) showed that the nAChE peak in chromatography was totally separate from all activity hydrolyzing naphthol and indoxyl esters (Vanha Perttula and Hopsu 1965a). Barron et al. (1966) have also found that the feline muscle ChEs are histochemically demonstrable with  $\alpha$ -NA but in quantitative studies their contribution is minimal. A preparation of human plasma cholinesterase has been shown to hydrolyze  $\alpha$ -NA in a biochemical system (Hopsu and Pontinen 1964). These discrepancies between biochemical and histochemical results cannot be solved without further studies.

A peculiar cerine and FCOO-resistant enzymic activity hydrolyzing thiocholine esters was found histochemically in or near the capillary endothelial cells. This activity was also found in the eluates of the starch gel as well as after DEAE cellulose chromatography as a separate peak. It was, however, not separable from an activity hydrolyzing ion-exchange naphthol esters and trifluoroacetyl  $\alpha$ -naphthylamide (Vanha Perttunen & Hopson 1965). This may correspond to the cerine-resistant activity in the rat hypothalamic blood vessel which hydrolyzed the same substrates but did not split 5-BIA (Holmes 1961).

The histochemical literature is full of observations of enzyme activity in different tissues and different cell types. Efforts have been made to study, e.g., tissue esterases both histochemically and after electrophoretic separation but an approach for the correlation between the results has remained inadequate (Eklund 1963, Fränkö et al 1964, Harkman 1964, Kokko 1965, Söderholm 1965). The present work shows that a large array of substrates and inhibitors as well as more than one fractionation method is required in order to get data enough for an appropriate correlation.

substrate this was also the best substrate for the bands 7-9. The second half of this peak which was more evident in the chromatography at pH 8.2 than at pH 7.0 (Vankha Perttula and Hopia 1963), hydrolysed  $\alpha$ -NB or  $\alpha$ -VA about equally as well as  $\alpha$ -NPr, while  $\alpha$ -VA seems to be even better as a substrate. This peak most probably corresponds to activity band 4 in starch gel electrophoresis and the peroxidic activity in the histochemical reaction. The esterolytic activity in the electrophoretic cells produced the same substrates as the peroxidic activity. In chromatography at pH 7.0 a small separate peak was found (Peak III Vankha Perttula and Hopia 1963) in the pH 8.2 chromatography it is only partly separated from the earlier peak. This peak has the same substrate specificity in addition to identical affector characteristics as the electrophoretic bands 5 and 6.

Band 1 differed from the other bands in substrate specificity.  $\alpha$ -NPr and  $\alpha$ -NB were the preferred substrates and no hydrolysis of  $\alpha$ -VA,  $\alpha$ -ASD and 5-BIA occurred. The activity was weaker than the other E and E bands and its counterpart in the chromatography was hidden behind the other peaks. Its histochemical localization is also uncertain. It might be a second enzyme species in the peroxidic cells or correspond to the weak parenchymal E and E activity in fluid gonadotropic cell.

The demonstration of AChE and nAChE activities in the histochemical work using *le*ThC and BuThC as substrates has been the preferred method using Gomori's modification (1952) of Kofler's method (1941). It has also been used for the demonstration of AChE and nAChE (Adams 1949, Holt 1952) and naphthol esters (Pepin and Pearse 1957, Pearse 1961) have also been used for the demonstration of AChE and nAChE differentiated from the nAChE by specific inhibitors. The nAChE activity in the rat pituitary thyrotropic cells was so weak that its contribution to the hydrolysis of these latter substrates could not be revealed. In quantitative homogenate studies with  $\alpha$ -VA as substrate the inhibition of the hydrolysis was minimal by eserine in starch gel electrophoresis and in quantitative studies using the method of Ellman et al (1961) showed that the nAChE peak in chromatography was totally separate from all activity hydrolysing naphthol and indoxyl esters (Vankha Perttula and Hopia 1963). Barron et al (1966) have also found that the feline muscle ChEs are histochemically demonstrable with  $\alpha$ -VA but in quantitative studies their contribution is minimal. A preparation of human plasma cholinesterase has been shown to hydrolyse  $\alpha$ -VA in a biochemical and histochemical results cannot be solved without further studies.

A peculiar esterase and P'600 resistant enzymic activity hydrolyzing thiocholine esters was found histochemically in or near the capillary endothelial cells. This activity was also found in the eluates of the starch gel as well as after DEAE cellulose chromatography as a separate peak. It was however not separable from an activity hydrolyzing long chained naphthol esters and trifluoroacetyl  $\alpha$  naphthylamide (Vanha Perttula and Hopsu 1965a). This may correspond to the esterase resistant activity in the rat hypothalamic blood vessels which hydrolyzed the same substrates but did not split 5 BIA (Holmes 1961).

The histochemical literature is full of observations of enzymic activities in different tissues and different cell types. Efforts have been made to study e.g., tissue esterases both histochemically and after electrophoretic separation but an approach for the correlation between the results has remained inadequate (Esila 1963, Iianko et al 1964, Harkonen 1964, Kokko 1965, Soderholm 1965). The present work shows that a large series of substrates and inhibitors as well as more than one fractionation method is required in order to get data enough for an appropriate correlation.

# QUANTITATIVE ESTIMATION OF CARBOXYLIC ESTERASES IN EXPERIMENTAL CONDITIONS

In the histochemical studies marked changes were observed in the enzymic reactions in different cells under different experimental conditions. Many factors may contribute to these changes. In fresh frozen sections the readily soluble  $\text{Er ns Es}$  were not demonstrated at all and in fixed tissue the  $\text{Es ns E}$  in gonadotropic and thyrotropic cells was almost totally inhibited by formalin. In addition the diazo salts may inhibit different enzymes to varying degrees. In order to avoid the drawback, esterase activity was estimated quantitatively from the pituitary homogenates. On the basis of the performed histochemical and fractionation studies four different substrates were chosen to be used in the quantitative studies:  $\alpha\text{-N}$  which is hydrolyzed by a large number of esterase components,  $\text{N 19}$  which is the preferred substrate for the  $\text{Er ns Es}$ ,  $\alpha\text{-NB}$  which is hydrolyzed only slightly by  $\text{Er ns Es}$  and demonstrates thus mainly  $\text{Fs ns E}$ ,  $\beta\text{-N P}$  which is mainly hydrolyzed by esterases having substrate preference to long chained naphthol esters.

## OWN INVESTIGATIONS

### Material and methods

The material consisted of 326 albino rats of the Sprague Dawley strain which were bred at the estate of the Pharmacy Orion at Mankkaa. Of them 196 were males and 130 were females. They were about 4 months of age at the beginning of the experiment. The animals were divided into 29 groups which were separately housed in cages. However the control groups were in six different cages. The number of animals and the treatment given are presented in Table I. Three subgroups received the same treatment but the time of treatment was different: group I for 10 days, group II for 30 days and group III for 90 days. All animals were fed *ad libitum* and fresh water was supplied each day for all groups except the adrenalectomized rats which received 0.9 per cent saline solution to drink. Table IV also gives the initial and final mean weights for each group.

The animals were killed by decapitation and some endocrine organ weights were measured. The pituitary gland was removed immediately, weighed first as a whole and again after removal of the posterior and intermediate lobes with a pin. After this each anterior pituitary gland was homogenized in 10 ml of distilled water. These samples were then stored in a refrigerator at  $+4^\circ\text{C}$ .

The substrates used were  $\alpha\text{-N}$ ,  $\alpha\text{-NB}$ ,  $\text{N 19}$  and  $\beta\text{-N P}$ . E600 (10 M) was included in the incubation medium for  $\text{N 19}$  hydrolysis. The hydrolysis rates of the

A peculiar eserine and U600 resistant enzymic activity hydrolyzing thiocholine esters was found histochemically in or near the capillary endothelial cells. This activity was also found in the eluates of the starch gel as well as after DEAE cellulose chromatography as a separate peak. It was however not separable from an activity hydrolyzing long chained naphthol esters and trifluoroacetyl  $\alpha$  naphthylamide (Vanha Perttula and Hopsu 1965a). This may correspond to the eserine resistant activity in the rat hypothalamic blood vessels which hydrolyzed the same substrates but did not split 5 BIA (Holmes 1961).

The histochemical literature is full of observations of enzymic activities in different tissues and different cell types. Efforts have been made to study e.g. tissue esterases both histochemically and after electrophoretic separation, but an approach for the correlation between the results has remained inadequate (Elli 1963 Lianko et al 1964 Harkonen 1964 Kolko 1965 Soderholm 1965). The present work shows that a large series of substrates and inhibitors as well as more than one fractionation method is required in order to get data enough for an appropriate correlation.

# QUANTITATIVE ESTIMATION OF CARBOXYLIC ESTERASES IN EXPERIMENTAL CONDITIONS

In the histochemical studies marked changes were observed in the enzymic reactions in different cells under different experimental conditions. Many factors may contribute to these changes. In fresh frozen sections the readily soluble  $\text{Er ns. Es}$  were not demonstrated at all and in fixed tissue the  $\text{Ns ns. E}$  in gonadotropic and thyrotropic cells was almost totally inhibited by formalin. In addition the diazo salts may inhibit different enzymes to varying degrees. In order to avoid these drawbacks, enzyme activity was estimated quantitatively from the pituitary homogenates. On the basis of the performed histochemical and fractionation studies four different substrates were chosen to be used in the quantitative studies:  $\alpha\text{-NA}$ , which is hydrolyzed by a large number of esterase components,  $\text{NAB}$  which is the preferred substrate for the  $\text{Er ns. Es}$ ,  $\alpha\text{-NB}$  which is hydrolyzed only slightly by  $\text{Er ns. Es}$  and demonstrates thus mainly  $\text{Es ns. Es}$ ,  $\beta\text{-NP}$ , which is mainly hydrolyzed by esterases having substrate preference to long chained naphthol esters.

## OWN INVESTIGATIONS

### Material and methods

The material consisted of 326 albino rats of the Sprague Dawley strain which were bred at the estate of the Pharmacy Orion at Mankkaa. Of them 196 were males and 130 were females. They were about 4 months of age at the beginning of the experiment. The animals were divided into 19 groups which were separately housed in cages. However the control groups were in six different cages. The number of animals and the treatment given are presented in Table I. Three subgroups received the same treatment, but the time of treatment was different: group I for 10 days, group II for 30 days and group III for 30 days. All animals were fed *ad libitum* and fresh water was supplied each day for all groups except the adrenalectomized rats which received 0.9 per cent saline solution to drink. Table I also gives the initial and final mean weights for each group.

The animals were killed by decapitation and some endocrine organ weights were measured. The pituitary gland was removed immediately weighed first as a whole and again after removal of the posterior and intermediate lobes with a pin. After this each anterior pituitary gland was homogenized in 10 ml of distilled water. These samples were then stored in a refrigerator at  $+4^\circ\text{C}$ .

The substrates used were  $\alpha\text{-NA}$ ,  $\alpha\text{-NB}$ ,  $\text{NAB}$  and  $\beta\text{-NP}$ . E600 (10 M) was included in the incubation medium for  $\text{NAB}$  hydrolysis. The hydrolysis rates of the



Table IX Initial and final body weights and the weights of the anterior pituitary gland

Experimental condition	Sex	No of animals	Duration of treatment (days)	Body
				Initial
Oil arach 0.1 ml sc	male	24	20	203 ± 1.4
Thyranon 0.2 % in food	male	10	10	210 ± 1.5
"      0.2 % in food	male	10	20	211 ± 8.9
"      0.2 % in food	male	11	30	194 ± 8.9
MTU 2.0 % in food	male	8	10	233 ± 8.0
"      2.0 % in food	male	10	20	208 ± 4.9
"      2.0 % in food	male	10	30	207 ± 11.1
Neo Hombreol 1.0 mg sc	male	9	10	230 ± 1.6
"      1.0 mg sc	male	10	20	219 ± 5.5
"      1.0 mg sc	male	10	30	186 ± 3.7
Castration	male	10	10	232 ± 9.5
"      "      "      "	male	9	20	213 ± 10.0
"      "      "      "	male	12	30	194 ± 6.4
Adreson 1.0 mg sc	male	8	10	225 ± 7.2
"      1.0 mg sc	male	10	20	239 ± 8.4
"      1.0 mg sc	male	10	30	187 ± 6.2
Adrenalectomy	male	10	10	235 ± 4.9
"      "      "      "	male	8	20	234 ± 7.7
"      "      "      "	male	7	30	219 ± 8.6
Oil arach 0.1 ml sc	female	22	20	189 ± 7.4
Progestin 1.0 mg sc	female	12	10	209 ± 4.9
"      1.0 mg sc	female	12	20	210 ± 4.8
"      1.0 mg sc	female	12	30	206 ± 10.1
Dimenformon 0.1 mg sc	female	11	10	215 ± 7.6
"      0.1 mg sc	female	11	20	221 ± 4.5
"      0.1 mg sc	female	13	30	197 ± 6.7
Castration	female	11	10	213 ± 4.4
"      "      "      "	female	13	20	233 ± 7.9
"      "      "      "	female	13	30	210 ± 4.9

Standard deviation

$p < 0.05$  }  
 $p < 0.01$  } Compared with controls  
 $p < 0.001$  }

substrates were measured as described in the preceding chapter. Details of the substrate concentrations and incubation times are given in Table VI. Duplicate enzyme assays were made with each substrate. Duplicate determinations of the protein content of the enzyme samples were made by a modified method for amide nitrogen (Stegemann 1958) in which the color after nesslerization was stabilized with KCN (Minari and Zilverman 1963). Each series of determinations was followed by appropriate standards of ammonium sulfate.

adrenal, testes or ovaries as well as the thyroid gland in the experimental series

weight (g)	Anterior pituitary gland (mg)	Adrenals (mg)	Testes vs ovaries (mg)	Thyroid gland (mg)
Final				
212 ± 10.5	57 ± 0.9	49.6 ± 10.1	2098 ± 183	91 ± 1.8
195 ± 9.6	48 ± 0.9 <sup>b</sup>	46.8 ± 5.8	3°38 ± 118	81 ± 2.2
186 ± 8.5	50 ± 0.6	50.1 ± 4.8	3157 ± 1°0	74 ± 1.8 <sup>b</sup>
168 ± 7.4	47 ± 0.8 <sup>b</sup>	54.1 ± 7.5	3069 ± 197	69 ± 1.4
249 ± 7.9	69 ± 1.0 <sup>b</sup>	46.5 ± 5.1	3709 ± 165 <sup>b</sup>	33.5 ± 5.3
235 ± 6.5	80 ± 1.1	59.0 ± 4.8	3335 ± 134	35.2 ± 7.2
237 ± 12.3	95 ± 0.7	36.8 ± 4.4	3331 ± 126	4°0 ± 6.1
°35 ± 9.4	51 ± 0.7	41.0 ± 9.8 <sup>b</sup>	2608 ± 118 <sup>b</sup>	
223 ± 4.8	49 ± 1.0	41.3 ± 4.3 <sup>b</sup>	2552 ± 76	
20. ± 5.3	45 ± 0.5	37.7 ± 8.0	2363 ± ■	
210 ± 7.7	63 ± 0.9	43.4 ± 6.6 <sup>b</sup>	—	
205 ± 8.6	67 ± 0.5 <sup>b</sup>	46.1 ± 4.5	—	
196 ± 7.3	70 ± 1.0	40.1 ± 5.5 <sup>b</sup>	—	
110 ± 7.5	56 ± 0.6	33.9 ± 3.2	2796 ± 168	
°0. ± 6.9	67 ± 0.7 <sup>b</sup>	3°4 ± 3.1	2519 ± 127	
184 ± 6.3	55 ± 0.8	23.0 ± 6.6	2435 ± 106	
114 ± 6.8	67 ± 1.2	—	2940 ± 78	
27 ± 8.4	70 ± 1.1 <sup>b</sup>	—	2801 ± 138	
30 ± 7.4	77 ± 1.6	—	3010 ± 98	
191 ± 6.6	95 ± 1.7	60.7 ± 9.5	53.2 ± 11.4	
212 ± 5.9	93 ± 1.4	61.8 ± 11.7	52.6 ± 8.8	
213 ± 6.9	94 ± 2.3	63.3 ± 8.1	62.2 ± 11.4	
194 ± 10.2	92 ± 1.8	61.3 ± 8.9	55.1 ± 10.3	
198 ± 6.5	16.5 ± 4.1	57.4 ± 9.4	61.5 ± 1°1 <sup>b</sup>	
104 ± 5.2	°5.8 ± 5.7	58.1 ± 11.2	81.1 ± 17.2	
189 ± 7.2	32.3 ± 10.2	60.7 ± 8.7	62.7 ± 11.6 <sup>b</sup>	
211 ± 5.9	95 ± 1.3	51.9 ± 8.7	—	
230 ± 6.5	11.5 ± 2.6	57.4 ± 6.3	—	
219 ± 4.3	10.3 ± 2.1	63.9 ± 11.3	—	

### Statistical treatment of the results

Standard deviations were calculated for the body and organ weights in the different groups using the conventional statistical formula. In determining the significance of the difference between two means Student's *t* test was applied. The probability *P* was obtained by comparing the calculated value of *t* with the tabulated values for *f* variab

In comparing the results of quantitative enzymic determinations the analysis

Table X Hydrolysis rate of  $\alpha$  naphthyl butyrate naphthol AS acetate with E600 (10<sup>-4</sup>M) different experimental conditions

Experimental condition		$\alpha$ Naphthyl butyrate m $\mu$ mole/ $\mu$ g N/min		Naphthol AS m $\mu$ mole/ $\mu$ g
		Mean of test group	Test groups combined	Mean of test group
Male control		3 829	3 829 $\pm$ 0 293	1 107
Thyranon	10 days	2 810		0 981
,	20 days	4 087	3 443 $\pm$ 0 197	1 242
,	30 days	3 434		1 160
MTU	10 days	3 418		0 910
,	20 days	6 092	4 857 $\pm$ 0 192	1 751
,	30 days	4 760		1 273
Neo Hombreol	10 days	5 588		1 298
,	20 days	5 998	3 810 $\pm$ 0 191 <sup>b</sup>	1 442
,	30 days	5 918		1 617
Castration	10 days	5 907		1 609
,	20 days	5 076	5 787 $\pm$ 0 187 <sup>b</sup>	1 744
,	30 days	6 178		1 623
Adreson	10 days	5 367		0 666
,	20 days	4 212	5 105 $\pm$ 0 191	0 682
,	30 days	5 787		0 816
Adrenalectomy	10 days	4 466		1 491
,	20 days	2 868	4 003 $\pm$ 0 198	1 046
,	30 days	4 637		1 563
Female control		4 778	4 778 $\pm$ 0 235	1 046
Progesterin	10 days	4 828		1 220
,	20 days	4 739	4 810 $\pm$ 0 145	1 172
,	30 days	4 993		1 078
Dimenformon	10 days	2 898		0 945
,	20 days	2 100	2 594 $\pm$ 0 146	1 000
,	30 days	2 401		0 961
Castration	10 days	6 112		1 153
,	20 days	6 421	6 576 $\pm$ 0 143 <sup>c</sup>	1 816
,	30 days	7 100		1 76

Standard error

$p < 0.05$  }  
 $p < 0.01$  } Compared with control\*  
 $p < 0.001$  }

variance was performed the within group variances and the between group variances were thus determined. The error risk probability for the variance ratio F was checked from the tabulated distribution for this test variable (Fisher and Yates 1953).

When comparing the significance of the difference between the mean of the individual test group and that of the control group the formula given for the t test was

naphthyl acetate and  $\beta$  naphthyl palmitate by the rat adenohypophyseal tissue homogenate in

rate min	$\alpha$ Naphthyl acetate m $\mu$ mole/ $\mu$ g N/min		$\beta$ Naphthyl palmitate m $\mu$ mole/ $\mu$ g N/10 min	
Test groups combined	Mean of test group	Test groups combined	Mean of test group	Test groups combined
1 107 $\pm$ 0 01a	1 950 1 348	1 950 $\pm$ 0 123	0 167 0 658	0 767 $\pm$ 0 109
1 130 $\pm$ 0 010	1 887 1 710 1 599	1 650 $\pm$ 0 082	1 045 0 706 1 153	0 819 $\pm$ 0 072
1 167 $\pm$ 0 010	3 448 2 433 2 842	2 555 $\pm$ 0 084	1 453 0 991 0 302	1 192 $\pm$ 0 074 <sup>b</sup>
1 457 $\pm$ 0 030	2 774 2 868 2 797	2 878 $\pm$ 0 087	0 236 0 344 0 214	2 294 $\pm$ 0 074 <sup>b</sup>
1 673 $\pm$ 0 010	2 709 3 273 2 036	2 056 $\pm$ 0 082	0 841 0 364 1 443	0 454 $\pm$ 0 072
2 773 $\pm$ 0 010	2 026 2 950 2 350	2 359 $\pm$ 0 094	0 490 0 624 0 551	0 799 $\pm$ 0 074
1 865 $\pm$ 0 010	1 936 2 666	2 327 $\pm$ 0 086	0 381 0 882	0 593 $\pm$ 0 077
1 046 $\pm$ 0 012	2 704 2 607	2 704 $\pm$ 0 115	0 592 1 295	0 592 $\pm$ 0 094
1 157 $\pm$ 0 007	2 507 2 558 1 616	2 547 $\pm$ 0 071	1 121 1 036 2 780	1 151 $\pm$ 0 058
0 070 $\pm$ 0 007	1 731 1 658 3 272	1 668 $\pm$ 0 012	2 492 0 398 0 597	0 532 $\pm$ 0 058
1 3 4 $\pm$ 0 007	3 162 3 432	3 290 $\pm$ 0 070	0 701 0 472	2 569 $\pm$ 0 057

applied. The term  $s^2$  is replaced by the square of the within group variance ( $s_1^2$ ) and the probability  $P$  is again obtained from the tabulated values of Student's  $t$  test.

In this investigation the difference between two means or in the variance ratio is said to be significant if  $0.01 < P \leq 0.05$  highly significant if  $0.001 < P \leq 0.01$  and very highly significant if  $P \leq 0.001$ .

Table X : Hydrolysis rate of  $\alpha$  naphthyl butyrate naphthol AS acetate with E600 (10+M) different experimental conditions

Experimental condition		$\alpha$ Naphthyl butyrate nmole/ $\mu$ g N/min		Naphthol AS nmol / $\mu$ g
		Mean of test group	Test groups combined	Mean of test group
Male control		3 829	3 829 $\pm$ 0 293	1 107
Thyranon	10 days	2 810		0 931
"	20 days	4 087	3 443 $\pm$ 0 18 <sup>a</sup>	1 742
"	30 days	3 434		1 183
MTU	10 days	3 418		0 910
"	20 days	6 092	4 852 $\pm$ 0 19 <sub>a</sub>	1 <sub>000</sub> <sup>a</sup>
"	30 days	4 760		1 213
Neo Hombreol	10 days	5 588		1 <sub>008</sub>
"	20 days	5 998	5 850 $\pm$ 0 191 <sup>b</sup>	1 44 <sup>a</sup>
"	30 days	5 938		1 617
Castration	10 days	5 957		1 669
"	20 days	5 076	5 787 $\pm$ 0 18 <sup>b</sup>	1 144
"	30 days	6 178		1 693
Adreson	10 days	5 367		0 666
"	20 days	4 312	5 10 <sub>0</sub> $\pm$ 0 192 <sup>b</sup>	0 68
"	30 days	5 787		0 816
Adrenalectomy	10 days	4 466		1 481
"	20 days	2 868	4 00 <sub>a</sub> $\pm$ 0 198	1 046
"	30 days	4 647		1 583
Female control		4 778	4 778 $\pm$ 0 235	1 046
Progestin	10 days	4 828		1 <sub>000</sub>
"	20 days	4 739	4 850 $\pm$ 0 145	1 172
"	30 days	4 983		1 078
Dimenformon	10 days	2 898		0 945
"	20 days	2 220	2 594 $\pm$ 0 146	1 00 <sup>a</sup>
"	30 days	2 401		0 965
Castration	10 days	6 112		1 153
"	20 days	6 421	6 576 $\pm$ 0 143	1 516
"	30 days	7 125		1 276

Standard error

$p < 0.05$   
 $p < 0.01$   
 $p < 0.001$

Compared with controls

variance was performed the within group variances and the between group variances were thus determined The error risk probability for the variance ratio F was checked from the tabulated distribution for this test variable (Fisher and Yates 1963)

When comparing the significance of the difference between the mean of the individual test group and that of the control group the formula given for the t test was

hyl acetate and  $\beta$  naphthyl palmitate by the rat adenohipophyseal tissue homogenate in

Test groups combined	$\alpha$ Naphthyl acetate nmole/ $\mu$ g N/min		$\beta$ Naphthyl palmitate nmole/ $\mu$ g N/10 min	
	Mean of test group	Test groups combined	Mean of test group	Test groups combined
7 $\pm$ 0.015	1.950 1.348	1.950 $\pm$ 0.123	0.767 0.658	0.767 $\pm$ 0.102
10 $\pm$ 0.010	1.897 1.710 1.599	1.650 $\pm$ 0.082	1.045 0.706 1.153	0.819 $\pm$ 0.072
5 $\pm$ 0.010	3.448 2.433 2.842	2.550 $\pm$ 0.084	1.473 0.991 0.302	1.192 $\pm$ 0.074
10 $\pm$ 0.010	2.774 2.868 2.787	2.828 $\pm$ 0.082	0.236 0.344 0.214	0.294 $\pm$ 0.074
7 $\pm$ 0.010	2.709 3.273 2.036	2.956 $\pm$ 0.082	0.841 0.364 1.443	0.454 $\pm$ 0.172
7 $\pm$ 0.010	2.026 2.950 2.350	2.359 $\pm$ 0.084	0.490 0.624 0.551	0.729 $\pm$ 0.17
5 $\pm$ 0.010	1.896 2.666	2.322 $\pm$ 0.086	0.381 0.882	0.118 $\pm$ 0.17
10 $\pm$ 0.012	2.704 2.607	2.704 $\pm$ 0.115	0.590 1.290	0.112 $\pm$ 0.17
5 $\pm$ 0.007	2.507 2.528 1.616	2.547 $\pm$ 0.071	1.121 1.036 0.780	1.121 $\pm$ 0.17
10 $\pm$ 0.007	1.731 1.658 3.072	1.668 $\pm$ 0.072	0.402 0.399 0.527	0.402 $\pm$ 0.17
10 $\pm$ 0.007	3.162 3.432	3.90 $\pm$ 0.070	0.701 0.412	0.701 $\pm$ 0.17

applied The term  $s^2$  is replaced by the square of the within-group variance and the probability  $P$  is again obtained from the tabulated values of  $F$ .  
In this investigation the difference between two means is said to be significant if  $0.01 < P \leq 0.05$ , highly significant if  $0.001 < P \leq 0.01$ , and very highly significant if  $P \leq 0.001$ .

## Results and discussion

Table IX gives the initial and final body weight of the animals in each individual group as well as the mean weights of the anterior pituitary glands, adrenals, testes or ovaries and the thyroid glands, the latter only in the control male group and after thyroxin and MTU treatment. The significance of the changes is given for the organ weights. The changes are in agreement with those previously reported in the literature under identical experimental conditions.

The mean hydrolysis rates of the four different substrates in the various test groups are given in Table X. The results show that in most of the experimental groups no apparent trend toward increasing or decreasing direction was found during the experimental intervals of 10, 20, and 30 days. For this reason the results of the three subgroups with identical treatment were combined and in each case treated as an entity, although the duration of the treatment was different.

When the esterolytic activity between the male and female control groups were compared it was found that the hydrolysis was significantly stronger in the female control group when  $\alpha$ NA or  $\alpha$ NB were used as substrates. No significant differences were, however, found in the hydrolysis of NAS and  $\beta$ NP.

The analysis of variance was separately performed for the male and female test groups in the experiments using the four different substrates. The results of the analysis are given in Tables XI—XIV and they show that there are very highly significant differences between the groups in the hydrolytic activities.

*$\alpha$ Naphthyl butyrate* — The hydrolysis of  $\beta$ NB was highly significantly increased after castration both in the male and female rats. This increase presumably represents partly the fine granular activity in both gonadotropic cells in fresh frozen sections and partly the pericytic activity in fixed sections. Estrogen treatment had a contrary effect on both these activities and this is shown as a very highly significant decrease in the quantitative determination. Progesterone was without effect on the total hydrolytic activity but testosterone had an increasing effect which presumably reflects the increased activity in FSH cells and STH cells. The simultaneous decrease of the esterase activity in LH cells has however an equalizing effect on the total hydrolytic activity.

Thyroxin treatment did not cause any significant change in the hydrolytic activity although in the TSH cells in fresh frozen sections a decrease of activity was evident. The increase after methylothiourea treatment

Table XI  $\alpha$  Naphthyl butyrate (nmole  $\alpha$  naphthol/ $\mu$ g N/min)

Male groups					
Analysis of variance					
Variation	Q	f	s	F	I
Between groups	155.79	6	25.965	13.49	<0.001
Within groups	363.76	189	1.92		
Total	519.55	195			
Female groups					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	296.40	3	99.470	78.84	<0.001
Within groups	152.56	126	1.211		
Total	448.96	129			

ment was significant and presumably due to the increased esterase activity in TSH cells in fresh frozen sections. The increase in the hydrolysis was also significant after cortisone treatment but the histochemical counterpart for this increase is difficult to point out. This increase may however, be due to the closer packing of the active gonadotropic cells after the shrinkage of the ACTH cells. No significant change was found after adrenalectomy.

*Naphthol-AS acetate* — In the total pituitary homogenate the E<sub>1</sub>s<sub>1</sub>N<sub>1</sub>E was found to represent only about 6% of the hydrolytic activity against N<sub>1</sub>AS (Table VII). E<sub>1</sub>r<sub>1</sub>N<sub>1</sub>L activity hydrolyzing N<sub>1</sub>AS was markedly increased after castration both in males and in females. The decrease after estrogen treatment was also very highly significant. Progesterone increased significantly this activity. These changes are in agreement with those observed histochemically in the gonadotropic cells.

No significant effect on the total hydrolysis of N<sub>1</sub>AS was obtained after thyroxine or MTU treatment. This is not in correlation with the histochemical changes in the E<sub>1</sub>r<sub>1</sub>N<sub>1</sub>L activity in the TSH cells but it is acceptable because of the simultaneous opposite change in the esterase activity in the STH cells. The increase after testosterone treatment is most probably in connection with the increased hydrolytic activity in STH cells.

Adrenalectomy caused a very highly significant increase while cortisone treatment markedly decreased N<sub>1</sub>AS hydrolysis. Histochemically identical changes were observed in the coarse granulated cytoplasmic activity in the ACTH cells.



Table XII Naphthol AS acetate + E600 ( $10^{-3}$  M) ( $m\mu$ mole naphthol AS/ $\mu$ g N/min)

Male groups					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	14.64	11	2.440	48.22	<0.001
Within groups	9.56	189	0.051		
Total	24.20	193			
Female group					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	5.48	3	1.827	56.39	<0.001
Within groups	4.08	126	0.032		
Total	9.56	129			

*$\alpha$  Naphthyl acetate* — This substrate is hydrolyzed by both E 3 ns E 3 and E 1 ns E 3 and changes in both categories are reflected in the hydrolysis. An increase was also obtained after castration both in males and females just as with the previously reported substrates. A very highly significant decrease was caused by estrogen treatment as was anticipated on the basis of the histochemical results. A marked increase was also found after methylthiouracil and testosterone treatment. Progesterone was without any significant effect while an increase was shown both after cortisone treatment and after adrenalectomy. These changes are relevant to the findings obtained with  $\alpha$  NB and N AS when the hydrolytic activities are combined.

Table VIII  $\alpha$  Naphthyl acetate ( $m\mu$ mole  $\alpha$  naphthol/ $\mu$ g N/min)

Male group					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	37.97	6	6.329	17.31	<0.001
Within groups	69.09	189	0.366		
Total	10.06	193			
Female groups					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	48.02	3	16.006	51.61	<0.001
Within groups	36.93	126	0.293		
Total	91.93	129			

Table XIV  $\beta$  Naphthyl palmitate ( $m\mu$ mole  $\beta$  naphthol/ $\mu$ g  $\backslash$ /10 min)

Male group					
Analysis of variance					
Variation	Q	f	s <sup>2</sup>	F	P
Between groups	14.42	6	2.403		
Within groups	53.36	189	0.283	8.33	<0.001
Total	67.78	195			
Female groups					
Analysis of variance					
Variation	Q	f	s <sup>2</sup>	F	P
Between groups	9.14	3	3.047		
Within groups	24.59	126	0.195	15.61	<0.001
Total	33.73	129			

Adrenalectomy caused only a slight increase in the hydrolysis of these substrates which was not significant but when both activities are partaking in the hydrolysis of  $\alpha$  NA, a significant change may be expected. After cortisone treatment the hydrolysis of  $\alpha$  NB was increased while that of NAS was decreased. Numerically the increase of the  $\Gamma$  s ns Es was greater and contributed also more markedly to the hydrolysis of  $\alpha$  NA.

**$\beta$  Naphthyl palmitate** — The most significant change in the hydrolysis of  $\beta$  NP was observed after progesterone treatment which caused a marked increase. Methylthouracil had also an increasing effect while a decrease was apparent after testosterone treatment and after castration in males. The hydrolysis in other groups was not significantly altered.

The site of the hydrolytic activities toward  $\beta$  NP separated by starch gel electrophoresis and DLAI cellulose chromatography could not be visualized in the histochemical study. The fractionation studies however, showed that the hydrolytic activities which split the short chained carboxylic esters contribute only weakly to the hydrolysis of  $\beta$  NP. Thus a different category of enzymes is mainly described in the quantitative studies. The results obtained in these studies also show that the changes obtained are not concomitant with  $\beta$  NP and those obtained with other substrates.

Table XII Naphthol AS acetate + E600 ( $10^{-3}$  M) ( $m\mu$ mole naphthol AS/ $\mu$ g N/min)

Male groups					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	14.64	6	2.440		
Within groups	9.56	189	0.051	48.22	<0.001
Total	24.20	195			
Female group					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	5.48	3	1.827		
Within groups	4.08	126	0.032	56.39	<0.001
Total	9.56	129			

*$\alpha$  Naphthyl acetate* — This substrate is hydrolyzed by both E s ns Es and E r ns Es and changes in both categories are reflected in the hydrolysis. An increase was also obtained after castration both in males and females just as with the previously reported substrates. A very highly significant decrease was caused by estrogen treatment as was anticipated on the basis of the histochemical results. A marked increase was also found after methylthiouracil and testosterone treatment. Progesterone was without any significant effect while an increase was shown both after cortisone treatment and after adrenalectomy. These changes are relevant to the findings obtained with  $\alpha$  NB and N AS when the hydrolytic activities are combined.

Table XIII  $\alpha$  Naphthyl acetate ( $m\mu$ mole  $\alpha$  naphthol/ $\mu$ g N/min)

Male groups					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	3.97	6	63.8		
Within groups	69.09	189	0.366	17.31	<0.001
Total	10.06	195			
Female groups					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	48.02	3	16.006		
Within groups	36.93	126	0.293	54.61	<0.001
Total	84.95	129			

Characteristic features for different cells have been searched also in inactive or incompletely differentiated stages with the histochemical enzyme reaction. Thus endocrine cells producing steroid hormones have been identified with a histochemical enzyme reaction although the hormones of these cells have not yet been demonstrated or the cells are still morphologically undifferentiated (Niemi and Ikonen 1962). The biosynthesis of the adenohypophyseal hormones includes, unfortunately, no such specific enzyme catalysis which could make the histoenzymological differentiation of the cells possible. In the oxidative metabolism of various cell types only minor differences have been found which show that the basophilic cells are usually most active but the other cells are not totally devoid of these activities (Bleicher et al 1961 Bilogh and Cohen 1962 Sobel 1964 Fand 1965).

There is a large number of esterases in different endocrine glands, their activity is quite intimately connected to hormone production (Allen 1957 Ikonen 1965 König 1965 Niemi et al 1966). Reports have also been published earlier on the esterase activity of the adenohypophyseal cells although quite different statements on their localization have been presented (Dumont 1956a, b Fand 1955, 1961 Pearse 1956 Lojda 1960 Lojda and Schreiber 1960, 1964, Sobel 1962 Jirasek 1963 Wächter and Pearse 1966). This at least partly can be explained due to species differences.

The present studies show that in the rat pituitary gland there is a positive correlation of the  $E_s$  and  $E$  activity to the production of FSH, LH, TSH and STH. The subcellular site of this reaction was not possible to specify with certainty. It was located evenly over the cytoplasm and solubilized by Triton X 100. The latter finding is the same as made by Carruthers and Baumler (1962) on the  $E_s$  and  $E$  in liver microsomes. The microsomal particles are known to be the site of intracellular protein synthesis. The role of this type of esterase in the glycoprotein and protein hormone synthesis cannot be specified due to the incomplete knowledge of the mechanism of hormone production. It may be assumed however that this type of carboxylic esterase does not directly act in the building up of a protein molecule although the hydrolytic enzymes are claimed to act as transferases (cf Hofster 1960). The site of the esterase activity in the microsomal membranes (Chaveau et al 1962) presupposes that these enzymes are somehow connected with the treatment of the raw material for hormone synthesis.

Another  $E_s$  and  $E$  activity in fresh frozen sections was mainly located in the STH cells where it was mostly confined to the cellular periphery. This type of esterase activity seems to be somewhat more intensely attached

to the intracellular particles, but can be dissolved also by Triton X 100 at higher concentrations. Part of this activity may reside in the microsomal particles but other cellular components including the cell membrane may be equally in question. STH cells in the active phase contain densely arranged lamellar endoplasmic reticulum in the periphery of the cell body (Herlant 1963, Yoshimura and Harumizu 1965). The secretory granules of these cells are transferred to the cell membrane and adhere to it; the limiting membrane is dissolved possibly by an intracellular enzyme activity (Farquhar 1961, Weiss 1965, Yoshimura and Harumizu 1965), which may be an esteroytic enzyme. After the release of the secretory granule out of the cell the granule membrane is left inside the cell and a ring of ribosomes is attached to it (Weiss 1965). These particles may thus also be responsible for the peripheral activity. In the gonadotropic cells the secretory granules lose their contents intracellularly and the dispersed material is claimed to be eliminated physicochemically (Yoshimura and Harumizu 1965).

Pearse (1956) reported that in fresh frozen sections of the human pituitary gland an intense esterase activity was found in the pericytic cells when indoxyl acetate was used as substrate. The present study shows that in the rat pituitary gland no such activity was observed but in formalin fixed sections the same cell type revealed a strong  $\text{E}_{\text{S-NB}}$  with  $\alpha\text{-NB}$  substrate but not with 5 BIA. The pericytic activity in the human pituitary was found to be quite variable (Pearse 1956) and this was also true in the rat pituitary gland. A positive correlation between this activity and the increase in the gonadotropic hormone secretion was however, clearly shown. The phagocytizing functions of these cells give reason to suppose that this soluble esterase activity is connected with the digestion processes of the engulfed lipid material. There is good reason to object to the idea of Pearse (1956) that this pericytic activity is derived from phagocytosis of the esterase bearing protein. Another esterase type was found however in the same cells after delivery for a short time period. This  $\text{E}_{\text{I-NB}}$  had the same characteristics as the intracellular coarse granulated activity in gonadotropic cells and it is supposed that this activity may be derived from the parenchymal cells after it has become useless for the cellular functions.

The role of the esterases in the secretory process of the pituitary gland could be better evaluated if the mechanism of the secretion would be worked out. Cardell (1962) working on *Triturus*, described buds of the parenchymal cells protruding during hormonal release, into the cytoplasm of either a pericapillary cell or a stellate pericytic cell. The bud is pinched

of to become a vacuole within the invaded cell. The cell then breaks down the granules and releases the hormone into its own cytoplasm from which it enters the pericapillary space. The same kind of budding was also found in the pituitary gland of the platyfish, but the secretory granule itself was released into the perivascular space and may move through the capillary endothelium or fenestrae in it into the capillary lumen in invisible form (Weiss 1965). The remnants of the parenchymal cell buds may, however, be acted upon by the perivascular cells and in the digestion of these cell components the perivascular esterase activity may equally well be involved.

A strong granular esterase activity in the mucoid cells of the paraffin embedded human hypophysis was found to reside in the Golgi zone (Pearse 1956) but its sensitivity to I 600 was not accurately specified. In the present study an I r ns E was confined to the same zone and a marked increase in this activity was connected with the increased secretory function. This effect was most intense in both gonadotropic cells after castration which is known to be connected with the increased content of FSH and LH in the pituitary gland (Greep and Chester Jones 1950, Cozens and Nelson 1961). An increase was also shown in FSH cells but not in LH cells after testosterone treatment which is in agreement with the dissociated action of this hormone on the secretory processes of FSH and LH (Hoogstra and Paesi 1957, Paesi et al 1953, 1959). A further confirmation for the contribution of this esterase to secretory function was obtained in young rats which had a large number of peripheral cells with an active microsomal P r ns E but were almost devoid of the I r ns E. These cells are probably already active in the synthesis of FSH but at this stage they do not release their hormones.

High F r ns E activity was shown in the Golgi area of the thyrotropic cells too. This finding is in agreement with that of Sobel (1962), who concluded that the actual site of this activity may be in the lysosomes. Both light and electron microscopic studies have confirmed the increase of the lysosomal particles in the Golgi area during augmented secretory activity of gonadotropic and thyrotropic cells (Yoshimura et al 1964, Yoshimura and Harumiya 1965).

A number of electron microscopic studies have shown that the Golgi apparatus is intimately connected with the formation of the secretory granules (Ichikawa 1959, Farquhar 1961, Gabe and Arvy 1961, Maillard 1963, Weiss 1965). The Golgi zone is not involved in the actual synthesis of the hormone but acts as a 'condensation center', where the granule membranes are possibly formed of the Golgi membranes. Novikoff et al

(1962) have suggested that the lysosomal hydrolases are involved in the condensation or other aspects of the secretory material accumulation

The fractionation studies showed that the total number of enzymic activities in the rat pituitary gland was greater than could be expected from the histochemical studies. Some of the enzyme double bands separated in the starch gel electrophoresis were quite similar in their inhibitor characteristics and substrate specificity. Those may be called 'isozymes' according to Markert and Møller (1959) or technical artefacts according to Cann (1966), until our growing knowledge of the multiple enzyme systems shows that these activities *in vivo* have really different cellular locations and differences also in characteristics other than their migration in electrophoresis.

Almost all the different esterase activities visualized in the starch gel slab could be correlated with separate activities in the histological sections. There were still other esterases more specific for the long chained naphthol esters that could not be demonstrated in sections, but were separated both in the starch gel electrophoresis and DEAE cellulose chromatography. The ignorance of the cellular site of these enzymes leaves their possible role unclear, although significant changes were found in the total activity using  $\beta$  NP as substrate. The quantitative studies with other substrates were properly in agreement with the changes found in histochemical studies during adequate experimental conditions.

## SUMMARY

The histology and the esterase activity of the rat anterior pituitary gland were studied in material from about 2000 animals. The production of pituitary hormones was altered in the following experimental conditions: castration, adrenalectomy, feeding with thyroxine and methylthiouracil, injection with testosterone, cortisone, progesterone, estrogen and reserpine, female rats during pregnancy and lactation and immature rats. To elucidate the histologically characteristic features of the six different parenchymal cell types as well as other cellular constituents, the following staining techniques were used: periodic acid-Schiff, orange G, aldehyde fuchsin, alcian blue at pH 3.0 and 0.2, tetrachrome, acid fuchsin, aniline blue, aldehyde thionin, and azocarmine orange G.

Carboxylic esterases were studied histochemically in fresh frozen and formalin fixed sections using acetyl and butyrylthiocholine as substrate for the demonstration of cholinesterases and  $\alpha$ -naphthyl acetate,  $\alpha$ -naphthyl propionate,  $\alpha$ -naphthyl butyrate,  $\alpha$ -naphthyl valerate,  $\alpha$ -naphthyl caprylate,  $\beta$ -naphthyl laurate,  $\beta$ -naphthyl myristate,  $\beta$ -naphthyl palmitate, naphthol AS acetate, naphthol AS D chloroacetate, 5-bromoindoxyl acetate and thioacetic acid for the demonstration of non-specific esterases. The esterases were classified with the aid of different substrates and modifier substances into four types: acetylcholinesterase (AChE), non-specific cholinesterase (nsChE), D600-sensitive non-specific esterase (Es nsE), and D600-resistant non-specific esterase (E1 nsE).

Carboxylic esterases of the total anterior pituitary homogenate were further fractionated by the following methods: separation into soluble and particle-bound components, starch gel electrophoresis and DEAE-cellulose column chromatography. The total esterase content of the homogenates was finally determined with a number of substrates and changes during some experimental conditions were elucidated.

### Histological and histochemical cell characteristics

*Somatotropic cells* were orangeophilic in the azocarmine orange G method, degranulated after methylthiouracil feeding and the most abun-



dant cell type in the young rats. The cellular periphery in fresh sections had an I s ns I, which increased during augmented hormone production and decreased after methylthiouracil treatment. In fixed sections a coarse granular perinuclear I r ns F was shown, which clearly increased in young rats and after testosterone treatment.

*Lactotropic cells* were carminophilic in the azocarmine-orange G method, erythrosinophilic in the tetrachrome method, most abundant during lactation and after estrogen and reserpine treatment and lacking in young rats. They showed negligible or no L s ns F and E r ns E even during augmented hormonal production.

*Corticotropic cells* contain erythrosinophilic granules in the tetrachrome method, they are present in large numbers after adrenalectomy, but almost totally absent after cortisone treatment. There was a weak peripheral E s ns F without any apparent change in experimental conditions, and a moderate coarse granular perinuclear activity in fixed sections, which increased after adrenalectomy.

*Thyrotropic cells* are polygonal, stain red with aldehyde fuchsin, blue with aldehyde-thionin, stain green with alcian blue at pH 0.2, and are located in the central part of the gland. Evenly distributed fine-granular cytoplasmic I s ns F increased in these cells after methylthiouracil feeding and decreased after thyroxine treatment. Coarse-granular perinuclear E r ns F increased also in fixed sections after methylthiouracil and decreased after thyroxine. These cells contained in addition a weak diffuse ns ChE with no apparent changes in experimental conditions.

*FSH cells* were round or oval with a perisinusoidal distribution in the peripheral parts of the gland including the 'sex zone', and lining the ventrocaudal edge of the gland in young rats. They were PAS-positive, aldehyde fuchsin negative, and violet stained in the alcian blue pH 0.2 method. Strong I s ns I was present in the whole cytoplasm even in young animals, increased after castration and testosterone treatment, was unaffected by progesterone and markedly decreased after estrogen. Strong coarse granular perinuclear I r ns I in fixed sections increased after castration and was unaffected by testosterone and progesterone, but was almost absent in the young rats.

*LH or ICSH cells* were round or oval perisinusoidal cells in the central part of the gland. They were PAS positive, aldehyde fuchsin negative, and red in the alcian blue pH 0.2 staining. Their number was markedly increased after castration and during pregnancy, and decreased after testosterone and progesterone. They were almost absent after estrogen treatment. Cytoplasmic fine granular E s ns F in these cells was greatly in-

creased after castration and during pregnancy, and decreased after testosterone progesterone and estrogen. Paraneuronal I r ns I in fixed sections was increased after castration and during pregnancy and absent after estrogen treatment.

*Percytic cells* were fusiform or stellate, blue stained with the acid fuchsin aniline blue method and intimately related to the gonadotropic cells after castration. No esterase activity was present in fresh frozen sections but in fixed sections a strong I r ns I was found with  $\alpha$  naphthyl butyrate as the preferred substrate. The activity was markedly increased during augmented secretory activity of the gonadotropic cells and absent after estrogen treatment. During the first days after delivery these cells in the female pituitary gland also have I r ns I probably derived from the paraneuronal cells.

*Capillary endothelial cells* showed a weak granular activity with thiocholine esters. It was not affected by the modifiers used and is consequently different from the esterases classified earlier. Weak I r ns I was shown only with acetic acid esters in fixed sections. No apparent changes were observed in either of these activities in the experimental conditions.

*Cleft epithelial cells* between the anterior and intermediate lobes showed a weak ns ChI with no apparent changes under the experimental conditions. The strong I s ns I in these cells in fixed sections was markedly increased after castration and adrenalectomy when also the holocrine secretion of these cells was increased.

## Fractionation of the esterases

*Soluble and particle bound enzymes* Both F r ns I's and I r ns I's were present in the soluble and particulate fractions after centrifugation of the pituitary homogenate in 0.25 M sucrose solution. A great portion of the particle bound activity was readily solubilized by Triton X 100, although some activity still remained in the particulate fraction. Activity which hydrolyzed  $\beta$  naphthyl palmitate was mainly particulate and only partially solubilized by Triton X 100.

*Starch gel electrophoresis* Eleven activity bands were shown when the starch slabs were stained by the histochemical esterase technique. Four of them were E r ns I's the others were E s ns E's. Most of the electrophoretic fractions could be identified with the activities demonstrated in the tissue sections. Two separate activities splitting  $\beta$  naphthyl palmitate and two main hydrolytic activities for thiocholine esters were found using

the eluates of the transversely cut starch gel blocks. One was not separable from the second  $\beta$  naphthyl palmitate activity, both were resistant to eserine and E600.

*DEAE cellulose column chromatography* Separation of the F1 and I peak from three partially separated E and E peaks was achieved. These activities were also separable from the nChE peak, one peak preferentially hydrolyzing long chained naphthol esters as well as from another peak splitting both thiocholine esters and long chained naphthol esters. Correlation was made with the previously separated proteolytic activities when the same method was used.

### Quantitative changes in total esterase activity

The changes in the hydrolysis of  $\alpha$  naphthyl acetate,  $\alpha$  naphthyl butyrate, naphthol AS acetate with E600 and  $\beta$  naphthyl palmitate in the experimental conditions (male rats: castrated or adrenalectomized, injected with testosterone or cortisone; fed with methylthiourea or thiouracine and female rats: castrated or injected with progesterone or oestrogen) were compared with male and female controls. Significant changes were obtained with all these substrates and they were generally in agreement with those obtained in the histochemical studies. Observations with  $\beta$  naphthyl palmitate could not be related to any histochemical results. The changes shown with this substrate were not analogous to those obtained with other substrates which is most probably due to the different enzyme categories in the hydrolysis of different substrates.

## REFERENCES

- ABE M, S P KRAMER and A M SCLIGMAN, The histochemical demonstration of pancreatic like lipase and comparison with the distribution of esterase *J Histochem Cytochem* 1964 10 364—363
- ADAMS D H The specificity of the human erythrocyte cholinesterase *Biochim Biophys Acta (Amst)* 1949 3 1—14
- ADAMS C W M and A G E IEARSE Classification of the mucoid (basophil) cells in the normal and pathological human adenohypophysis *J Endocr* 1959 18 147—153
- ADAMS C W M and K V SWETTERMAN, The histochemical identification of two types of basophil cells in the normal human adenohypophysis *J Path Bact* 1958 75 95—103
- ALDRIDGE W V The differentiation of true and pseudo cholinesterase by organophosphorus compounds *Biochem J* 1953a 53 62—67
- ALDRIDGE W V, Serum esterases I Two types of esterase (A and B) hydrolysing p-nitrophenyl acetate propionate and butyrate and a method for their determination *Biochem J* 1953b 53 110—117
- ALDRIDGE W V Serum esterases II An enzyme hydrolysing diethyl p nitrophenyl phosphate (E800) and its identity with the A esterase of mammalian sera *Biochem J* 1953c 53 117—123
- ALDRIDGE W V and V K JOHNSON, Cholinesterase succinic dehydrogenase, nucleic acids esterase and glutathione reductase in subcellular fractions from rat brain *Biochem J* 1959 73 270—276
- ALLEN J A chemical and histochemical study of aliesterase in mouse adrenal cortex under hormonal influences and altered sodium intake *Endocrinology* 1957 61 368—374
- ALLEN S L J M ALLEN and B M LICHT Effects of Triton X 100 upon the activity of some electrophoretically separated acid phosphatases and esterases *J Histochem Cytochem* 1965 13 431—440
- ALLEN, J M and R L HILVER, A histochemical study of enzymes in the epididymis of normal castrated and hormone replaced castrated mice separated by zone electrophoresis *J Histochem Cytochem* 1960 8 50—57
- ARVY L, Données histoenzymologiques sur l'hypophyse de *Bos taurus* L. *C R Soc Biol (Paris)* 1961 155 2119—2122
- ARVY L Contribution à l'étude des activités enzymatiques dans l'hypophyse de *Equus caballus* L. (Mammifères Périssodactyles) *C R Soc Biol (Paris)* 1962 156 1758—1759
- ARVY L *Histo enzymologie des Glandes Endocrines* Gauthier Villars Paris 1963
- ARVY L Contribution à l'histoenzymologie comparée de l'hypophyse In *Proc 5th Int Neurochem Symp* Pergamon Press Oxford London New York Paris 1964 313—322

- APVY, L. and F. DU MESNIL DU BUISSON, Histochemistry of the hypophysis of *Sus scrofa* L. ♂ var domestique *C R Soc Biol (Paris)* 1961 155 2123—2126
- AUGUSTINSSON, K. B., Butyryl and propionylcholinesterases and related types of esterolytic enzymes. In *The Enzymes* Vol 4A Academic Press New York and London 1960 521—540
- BAKER, B. L., and N. B. EVERETT, The effect of small doses of diethylstilbestrol on the anterior hypophysis of the immature rat *Endocrinology* 1944 34 254—264
- BALOGH, K. JR. and E. B. COMEN, Histochemical localization of oxidative enzyme systems in the human anterior pituitary *Endocrinology* 1962 70 874—879
- BARNES, B. G., Electron microscope studies on the secretory cytology of the mouse anterior pituitary *Endocrinology* 1962 71 618—628
- BARNETT, P. J., The distribution of esterolytic activity in the tissues of the albino rat as demonstrated with indoxyl acetate *Anat Rec* 1952 114 511—599
- BARNETT, R. J. W. D. ROTH, and J. SALZER, The histochemical demonstration of the sites of luteotropic hormone in the rat pituitary gland *Endocrinology* 1961 69 1047—1059
- BARON, H. D., J. BERNSON, and A. P. HESS, Esterases and proteins of normal and atrophic feline muscle *J Histochem Cytochem* 1966 14 1—24
- BAYLIS, B. J., and A. TODRICK, The use of a selective acetylcholinesterase inhibitor in the estimation of pseudocholinesterase activity in rat brain *Biochem J* 1956 62 62—67
- BENOIT, J. and C. DA LAKE, *Cytologie de l'Adenohypophyse* Colloq Int Centre Natl Rech Sci (Paris) 1963
- BERGMAN, F., and S. RIMON, The effect of pH variations on the activities of Cesterase *Biochem J* 1959 70 339—344
- BERGMAN, F. and S. RIMON, Fractionation of Cesterase from hog's kidney extract *Biochem J* 1960 77 209—214
- BERGMAN, F., R. SEGAL, and S. RIMON, A new type of esterase in hog kidney extract *Biochem J* 1957 67 481—486
- BERSON, J. K. D. BARON, and H. NORGELLO, Bound and soluble esterases in human brain *Biochem J* 1964 91 24C—26C
- BLECHER, S. M. KARNOVSKY and N. FREINKEL, Histochemical topography of anterior pituitary: correlations between enzyme activities and endocrine function of individual cells *J Clin Invest* 1961 40 1024
- CANN, J. P., Multiple electrophoretic zones arising from protein buffer interaction *Biochemistry* 1966 5 1108—1112
- CAPPELL, D. F., Intravital and supravital staining II. Blood and organs *J Path Bact* 1929 36 679—674
- CARDELL, R. I. JR., Observations on the microstructure of the salamander pituitary gland. Doctoral Thesis Univ of Virginia Charlottesville 1962
- CARDELL, R. I. JR., Ultrastructure of the salamander thyroidectomy cell *J Ultrastruct Res* 1964 10 515—52
- CAPRUTHERS, C. and A. BAUMLER, Esterase distribution in mouse liver *Arch Biochem Biophys* 1961 94 351—357
- CAPRUTHERS, C. and A. BAUMLER, The influence of various detergents on the esterase and glucose 6 phosphate activities of mouse liver microsomes *Arch Biochem Biophys* 1962 99 459—465

- CARRUTHERS, C, E HEINS, and A BALMER Fractionation of mouse liver microsome esterases *Canad J Biochem* 1965 43 97—104
- CATCHPOLE H R Distribution of glycoprotein hormones in the anterior pituitary gland of the rat *J Endocr* 1949 6 218—225
- CHAVEAU J, Y MOUTÉ C ROUILLET, and J SCHNEEBELI Isolation of smooth vesicles and free ribosomes from rat liver microsomes *J Cell Biol* 1969 12 17—29
- CONSOLANDI, G and G BRIZIARELLI Le Mastzellen dell'ipofisi umana *Lat Ist Anat Univ Perugia* 1952 1<sup>a</sup> 21—55
- COZENS D A and M M NELSON Effects of ovariectomy on the follicle stimulating and interstitial cell stimulating content of the anterior pituitary of the rat *Endocrinology* 1961 68 767—772
- CRUPI, S B Osservazioni sulla lipolisi ipofisaria (Topografia endocellulare della lipasi) *Riv Anat pat* 1956 10 974—978
- CUSHING, H and L M DAVIDOFF The pathological findings in four autopsied cases of acromegaly with a discussion of their significance *Pockefeller Inst Med Res Monogr* 1927 22 1—131
- DAWSON A B The demonstration of differential staining of two types of acidophiles in the anterior pituitary gland of the rat *Anat Rec* 1954 120 810
- DAWSON A B, The occurrence of two tinctorial types of acidophiles (carminophiles and orangeophiles) in the pars distalis of the mammalian pituitary gland *Anat Rec* 1963 145 315
- DAWSON A B and H M FRIEDGOOD Differentiation of two classes of acidophiles in the anterior pituitary of the female rabbit and cat *Stain Technol* 1938 13 17—21
- DELLA CORTE F and A. BIONDI, The localization of the fluorescent anti FSH globulin in the pituitary of pig (*Sus scrofa* L var *domestica* Gray) *Fit Biol* 1964 57 369—376
- DEOM G F BURKERT and H. U TIETZE Thyreoidektomiezellen im Hypophysen vorderlappen der Ratte nach Nebennierenblockade Zur Frage der Bildungsstätte des ACTH *Z Zellforsch* 1962 57 679—691
- DEOM B and H U TIETZE Zur experimentellen Histophysiologie der  $\beta$  Zellen der Rattenhypophyse nach Untersuchungen mit der Peramaisensaure Alcianblau Reaktion *Endocrinologie* 1962 42 284—294
- DIEGENBACH, P C Use of inhibitors in cholinesterase histochemistry *Nature (Lond)* 1965 207 303
- DUBOIS P and E GIROD Nouvelles recherches sur l'ultrastructure des cellules gonadotropes de l'hypophyse antérieure chez le Hamster dore (*Mesocricetus auratus* Waterh) castré *C R Soc Biol (Paris)* 1964 158 2102—2106
- DUMONT L Localisation histochimique d'acétylcholinestérase dans l'adénohypophyse du Lapin *C R Acad Sci (Paris)* 1956a 242 296—298
- DUMONT L Localisation histochimique de la cholinestérase dans l'adénohypophyse du Cobaye *C R Soc Biol (Paris)* 1956b 120 728—732
- DE DUVE C Lysosomes a new group of cytoplasmic particles In *Subcellular Particles* The Ronald Press Company New York 1959 1 8—159
- ELFTMAN H Aldehyde fuchsin for pituitary cytochemistry *J Histochem Cytochem* 1959 7 98—100
- ELLMAN G L K B COURTNEY V ANDRES JR and H M FEATHERSTONE A new and rapid colorimetric determination of acetylcholine esterase activity *Biochem Pharmacol* 1961 7 88—95

- EMMART, E W, S S SPICER, and R W BATES Localization of prolactin within the pituitary by specific fluorescent antiprolactin globulin *J Histochem Cytochem* 1963 11 365-373
- ERANKO O, M HARKONEN, A KOKKO and L IIVINEN Histochemical and starch gel electrophoretic characterization of desmo- and lyso esterases in the sympathetic and spinal ganglia of the rat *J Histochem Cytochem* 1964 12 570-581
- ERANKO O, A KOKKO and U SODERHOLM Histochemical evidence of three types of esterases in the adrenal medulla of the rat *Histochemie* 1964 2 383-389
- ERANKO, O, A KOKKO and U SODERHOLM Starch electrophoretic separation of esterases in the adrenal medulla adrenal cortex and the brain of the rat and the hamster *Ann Med exp Fenn* 1962b 40 146-161
- ESILA R Histochemical and electrophoretic properties of cholinesterases and non specific esterases in the retina of some mammals including man *Acta ophthalm (Kbh)* 1963 Suppl 77
- EZPIN C, and A MURRAY, The cells of the human adenohypophysis in pregnancy thyroid disease and adrenal cortical disorders In *Cytologie de l'Adenohypophyse Colloq Int Centre Natl Rech Sci (Paris)* 1963 128 183-199
- FAND, S B The histochemistry of the human pituitary body I Preliminary report including a differentiation between bromophils of the anterior and posterior lobes *J clin Endocr* 1960 15 695-692
- FAND S B, Glandular epithelial cells in the human pars nervosa histochemistry distribution classification and possible clinical significance *Amer J clin Path* 1961 35 210-221
- FAND S B, Demonstration of  $\alpha$  glycerophosphate dehydrogenase in the human pituitary gland with special reference to the cells of the pars tuberalis *Endocrinology* 1960 73 30-324
- FARQUHAR M G, Corticotrophs of the rat adenohypophysis as revealed by electron microscopy *Anat Rec* 1967 127 291
- FARQUHAR M G Origin and fate of secretory granules in cells of the anterior pituitary gland *Trans N Y Acad Sci* 1961 23 346-361
- FARQUHAR M G, and J F LINFHART Cytologic alterations in the anterior pituitary gland following thyroidectomy: an electron microscope study *Endocrinology* 1964 55 857-876
- FERRER J Histophysiologia of the pituitary cleft and colloid cysts in the adenohypophysis of the rat Changes after gonadectomy and adrenalectomy *J Endocr* 1966 13 349-353
- FIELD E J The effects of adrenalectomy on the anterior lobe of the pituitary gland of the rat *J Anat (Lond)* 1958 92 131-142
- FINERTY J C, M HESS and R BIKHAMMER Pituitary cytological manifestations of heightened adrenocorticotrophic activity *Anat Rec* 1962 114 115-124
- FISHER A W F and D BULMER Studies of differential staining with acid dyes in the human adenohypophysis *Quart J micr Sci* 1964 105 461-473
- FISHER R A and F YATES *Statistical Tables for Biological Agricultural and Medical Research* Oliver and Boyd London 1963
- GABE, M and L ARVY Gland cells In *The Cell* Vol 1 Academic Press New York London 1961 1-88
- GARVIE W H H Mast cells of the pituitary gland *Nature (Lond)* 1960 205 1334
- GIRARD G P Etudes sur la localisation cytologique de la fonction corticotrope anté hypophysaire *Path et Biol* 1964 12 1244-1262

- GIROD C P Dubol and M Curé Etude au microscope électronique des cellules gonadotropes antéhypophysaires du Hamster doré (*Mesocricetus auratus* Waterh) intact ou castré *C R Acad Sci (Paris)* 1961 258 6336-6339
- GLENBER, G G, V K HOPSL and L A COHEN, Histochemical demonstration of a trypsin like esterase activity in mast cells *J Histochem Cytochem* 1961 10 109-110
- GOMORI, G The microtechnical demonstration of sites of lipase activity *Proc Soc exp Biol (NY)* 1945 58 362-364
- GOMORI G *Microscopic Histochemistry Principles and Practice* The University of Chicago Press Chicago 1951
- GOMORI G Histochemistry of human esterase *J Histochem Cytochem* 1955 3 479-494
- GREEN, J D, Comparative anatomy of hypophysis with special reference to its blood supply and innervation *Amer J Anat* 1951 88 25-311
- GREEN R O, and I CHESTER JONES, Steroid control of pituitary function *Recent Progr Hormone Res* 1950 5 197-261
- GRIESBACH, W E Über die Darstellung von zwei Typen von Basophilen in der Rattenhypophyse (Ein Beitrag zur Lokalisation der Hypophysenhormone) *Klin Wochr* 1953 31 926-930
- GROSVENOR, C E, and C W TURNER Pituitary heterogenic hormone concentration and milk secretion in lactating rats *Endocrinology* 1959 65 515-539
- GRUMBACH, M M, Intracellular detection of hormones by immunochemical means Growth hormone *Ciba Found Coll Endocr* 1961 14 33-374
- HALMI N S Two types of basophils in the anterior pituitary of the rat and their respective cytophysiological significance *Endocrinology* 1950 47 289-299
- HALMI N S Differentiation of two types of basophils in the adenohypophysis of the rat and the mouse *Stain Technol* 1951a 26 61-64
- HALMI N S Two types of basophils in the rat pituitary 'thyrotrophs and gonadotrophs' vs beta and delta cells *Endocrinology* 1952b 50 140-142
- HALMI N S and E M BOGDANOV Effect of thyroidectomy on ACTH content of rat adenohypophysis *Proc Soc exp Biol (NY)* 1951 78 518-520
- HANSON V and G TOSCHI Electron microscopy of microosomal fractions from rat brain *Exp Cell Res* 1959 16 256-271
- HARRIS G W *Neural Control of the Pituitary Gland* Arnold London 1955
- HELLBAUM A A L C McARTHUR P J CAMPBELL and J C FIVERTY, The physiological fractionation of pituitary gonadotropic factors correlated with cytological changes *Endocrinology* 1961 69 144-153
- HERLANT M Les granulations acidophiles de l'hypophyse et leur signification physiologique *C R Soc Biol (Paris)* 1952 146 1230-1232
- HERLANT M, Etude critique de deux techniques nouvelles destinées à mettre en évidence les différentes catégories cellulaires présentes dans la glande pituitaire *Bull Microscopie appl* 1960a 10 37-43
- HERLANT M Cytochimie du lobe antérieur de l'hypophyse *Anat An* 1960b 109 (Ergbd) 562-596
- HERLANT, M, Apport de la microscopie électronique à l'étude du lobe antérieur de l'hypophyse In *Cytologie de l'Adenohypophyse Colloq Int Centre Natl Rech Sci* (Paris) 1963 129 73-90
- HERLANT M The cells of the adenohypophysis and their functional significance *Int Rev Cytol* 1964a 17 299-381



- EMMART, E W & S SPICER and R W BATES Localization of prolactin within the pituitary by specific fluorescent antiprolactin globulin *J Histochem Cytochem* 1963 11 365-373
- EPANKO, O, M HAPEDAKEN A KOKKO, and L PAISANEN Histochemical and starch gel electrophoretic characterization of de mo and lyo esterases in the sympathetic and spinal ganglia of the rat *J Histochem Cytochem* 1964 12 570-581
- EPANKO, O, A KOKKO and U SÖDERHOLM Histochemical evidence of three types of esterases in the adrenal medulla of the rat *Histochemie* 1964 2 383-398
- EPANKO, O, A KOKKO, and U SÖDERHOLM, Starch electrophoretic separation of esterases in the adrenal medulla adrenal cortex and the brain of the rat and the hamster *Ann Med exp Fenn* 1962 40 146-161
- ESILA, R Histochemical and electrophoretic properties of cholinesterases and non specific esterases in the retina of some mammals including man *Acta ophthal (Kbh)* 1963 Suppl 77
- FZPIN, C, and S MURRAY The cells of the human adenohypophysis in pregnancy thyroid disease and adrenal cortical disorders In *Cytologie de l'Adenohypophyse Colloq Int Centre Natl Rech Sci (Paris)* 1963 128 183-199
- FAND, S B, The histochemistry of the human pituitary body I Preliminary report including a differentiation between ba ophils of the anterior and posterior lobes *J clin Endocr* 1965 15 645-69.
- FAND S B, Glandular epithelial cells in the human pars nervosa histochemistry distribution classification and possible clinical significance *Amer J clin Path* 1961 35 210-221
- FAND S B, Demonstration of a glycerophosphate dehydrogenase in the human pituitary gland with special reference to the cells of the pars tuberalis *Endocrinology* 1965 77 320-324
- FARQUHAR M G Corticotrophs of the rat adenohypophysis as revealed by electron microscopy *Anat Rec* 1967 127 291
- FARQUHAR M G, Origin and fate of secretory granules in cells of the anterior pituitary gland *Trans N Y Acad Sci* 1961 23 346-351
- FARQUHAR M G and J F PINFILIAT Cytological alterations in the anterior pituitary gland following thyroidectomy an electron microscope study *Endocrinology* 1964 65 827-876
- FREPER, J, Histophysiology of the pituitary cleft and colloid cysts in the adenohypophysis of the rat Changes after gonadectomy and adrenalectomy *J Endocr* 1966 13 349-353
- FIELD E J The effects of adrenalectomy on the anterior lobe of the pituitary gland of the rat *J Anat (Lond)* 1958 92 137-142
- FIVERTY J C M HESS and M BINHAMMER Pituitary cytological manifestations of heightened adrenocorticotrophic activity *Anat Rec* 1962 114 115-124
- FISHER A W F and D BULMPP Studies of differential staining with acid dyes in the human adenohypophysis *Quart J micr Sci* 1964 105 467-473
- FISHER R A and F YATES *Statistical Tables for Biological Agricultural and Medical Research* Oliver and Boyd London 1963
- GABE M and L ARVI Gland cells In *The Cell* Vol 1 Academic Press New York London 1961 1-89
- GARVIE W H H Mat cells of the pituitary gland *Nature (Lond)* 1965 205 1334
- GIBOD C Recherches sur la localisation cytologique de la fonction corticotrope anté hypophysaire *Path et Biol* 1964 12 1244-1265

- GIROD C P Dubois and M Curé Etude au microscope électronique des cellules gonadotropes antéhypophysaires du Hamster doré (*Mesocricetus auratus* Waterh) extrait du castré C P Acad Sci (Paris) 1964 258 6336—6338
- GLENNER G G, V K HORSE and L A COMPTON, Histochemical demonstration of a trypsin like esterase activity in mast cells J Histochem Cytochem 1962 10 109—110
- GOMORI, G The microtechnical demonstration of sites of lipase activity Proc Soc exp Biol (N Y) 1945 58 362—364
- GOMORI G Microscopic Histochemistry Principles and Practice The University of Chicago Press Chicago 1950
- GOMORI G, Histochemistry of human esterase J Histochem Cytochem 1955 3 419—484
- GREEN J D Comparative anatomy of hypophysis with special reference to its blood supply and innervation Amer J Anat 1931 33 233—311
- GREEN, R O, and I CHESTER JONES Steroid control of pituitary function. Recent Progr Hormone Res 1956 5 197—261
- GRUBBACH W E Über die Darstellung von zwei Typen von Basophilen in der Patten hypophyse (Ein Beitrag zur Lokalisation der Hypophysenhormone) Klin Wochr 1953 31 926—930
- GROSVENOR O E, and C W TURNER, Pituitary lactogenic hormone concentration and milk secretion in lactating rat Endocrinology 1959 63 513—539
- GRUMBACH, M M, Intracellular detection of hormones by immunochemical means Growth hormone Ciba Found Coll Endocr 1962 14 313—374
- HALMI N S Two types of basophils in the anterior pituitary of the rat and their respective cytophysiological significance Endocrinology 1950 47 289—299
- HALMI N S Differentiation of two types of basophils in the adenohypophysis of the rat and the mouse Stain Technol 1952a 27 61—64
- HALMI N S Two types of basophils in the rat pituitary thyrotrophs and gonadotrophs vs beta and delta cells Endocrinology 1952b 50 140—143
- HALMI N S and E M BOGDANOVIC Effect of thyroidectomy on ACTH content of rat adenohypophysis Proc Soc exp Biol (N Y) 1951 78 519—520
- HANZOV V and G TOSCHI Electron microscopy of microosomal fractions from rat brain. Exp Cell Res 1959 16 256—261
- HARPER G W Neural Control of the Pituitary Gland Arnold London 1955
- HELLBAUM A A L G McARTHUR P J CAMPBELL and J C FINERTY The physiological fractionation of pituitary gonadotropic factors correlated with cytological changes Endocrinology 1961 68 144—153
- HERLANT, M, Les granulations acidophiles de l'hypophyse et leur signification physiologique C P Soc Biol (Paris) 1952 146 1780—1782
- HERLANT, M Etude critique de deux techniques nouvelles destinées à mettre en évidence les différentes catégories cellulaires présentes dans la glande pituitaire Bull Microscopie 1960a 10 37—44
- HERLANT M Cytochimie du lobe antérieur de l'hypophyse Anat An 1960b 109 (Ergbd) 562—596
- HERLANT M Apport de la microscopie électronique à l'étude du lobe antérieur de l'hypophyse In Cytologie de l'Adenohypophyse Colloq Int Centre Natl Rech Sci (Paris) 1963 153 7—90
- HERLANT M The cells of the adenohypophysis and their functional significance Int Rev Cytol 1964a 17 299—380

- HERLANT M Present state of knowledge concerning the cytology of the anterior lobe of the hypophysis In *Proc 2nd Int Cong Endocr Excerpta Med Found Int Cong Ser* 1964h 23 468—491
- HERLANT, M and B GRIGNON, Le lobe glandulaire de l'hypophyse chez la Tortue terrestre (*Testudo mauritanica* DuRoi) Etude histochemique et histophysiologique *Arch Biol (Liege)* 1961 72 97—151
- HERLANT M and J KLATERSKY Etude au microscope électronique des cellules corticotropes de l'hypophyse *C R Ass Anat* 1963 118 720—729
- HERMANUS, J P J L PASTEELS and M HERLANT Localisation cellulaire de la sécrétion d'interméline dans l'hypophyse de foetus humain en culture de tissus *C R Acad Sci (Paris)* 1964 258 6530—6532
- HESS R and A G E PEARSE, The histochemistry of indoxylesterase of rat kidney with special reference to its cathepsin like activity *Brit J exp Path* 1959 39 292—299
- HILDEBRAND J E E G RENVELS, and J C FINEPTY, Gonadotrophic cells of the rat hypophysis and their relation to hormone production *Z Zellforsch* 1957 46 400—411
- HOFSTEE H J Fatty acid esterases of low eserine sensitivity and related enzymes In *The Enzymes* Vol 4 A Academic Press, New York and London 1960 495—500
- HOLMES R L, Esterases of the hypothalamo hypophyseal system In *The Cytology of Various Tissue* Taylor and Francis London 1961 1—4
- HOLMSTEDT B A modification of the thiocholine method for the determination of cholinesterase I Biochemical evaluation of selective inhibitors *Acta physiol scand* 1957 40 322—330
- HOLT, S J A new principle for the histochemical localization of hydrolytic enzymes *Nature (Lond)* 1952 169 271—273
- HOLT, S J, The value of fundamental studies of staining reactions in enzyme histochemistry, with reference to indoxyl methods for esterases *J Histochem Cytochem* 1956 4 541—554
- HOLT, S J Indigogenic staining methods for esterases In *General Cytochemical Methods* Vol 1 Academic Press New York and London 1958 375—398
- HOLT S J, Some observations on the occurrence and nature of esterases in lysosome In *Ciba Foundation Symposium Lysosomes* Churchill London 1963 114—120
- HOLT S J and R P J WITTIERS, Studies on enzyme cytochemistry V An appraisal of indigogenic reactions of esterase localization *Proc Roy Soc (Biol)* 1959 118 520—532
- HOOGSTRA, M J and F J A. PARES The F S H content of the hypophysis of the rat as influenced by androgen (with additional data on the effect of oestrogen and of combined oestrogen testosterone treatment) *Acta endocr (Kbh)* 1957 24 353—360
- HOPFU, V K. and G G GLENNEK Further observations on histochemical esterase and amidase activities with similarities to trypsin *J Histochem Cytochem* 1963a 11 520—528
- HOPFU V K., and G G GLENNEK A histochemical enzyme kinetic system applied to the trypsin like amidase and esterase activity in human mast cells *J Cell Biol* 1963b 17 503—510
- HOPFU, V K and G G GLENNEK Characterization of enzymes hydrolyzing acyl naphthylamides I Mono and dihalogen derivatives *J Histochem Cytochem* 1964 12 674—686

- HORST, V. K. and J. PÜNTING, The motor end plate reaction with naphthol AS  $\alpha$  aminocaproate *J Histochem Cytochem* 1964 12 857-864
- HORSU, V. K. R. SAKITI, and G. G. GLENNER Enzymic hydrolysis of acyl naphthyl amides in liver tissue *Ann Med exp Fenn* 1963 43 106-113
- HORSU, V. K. R. SAKITI, and G. G. GLENNER, Characterization of enzymes hydrolyzing acyl naphthylamides II Trihalogen derivatives *J Histochem Cytochem* 1963b 13 117-124
- HUNTER, R. L., and M. E. BURSTONE The xymogram as a tool for the characterization of enzyme substrate specificity *J Histochem Cytochem* 1960 8 58-62
- HUNTER, R. L. and C. L. MARKEET Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels *Science* 1957 125 1204-1205
- HURST, V., and C. W. TURNER Lactogenic hormone content of anterior pituitary gland of albino mouse as compared to other species *Endocrinology* 1942 31 334-339
- HÜLSMANN, W. C. A. J. M. FABRIS and H. DE JIJTER Inhibition of lipase activity of lung and adipose tissue by phenothiazine derivatives. *Nature (Lond)* 1964 202 1336-1337
- HYYPÄ, M. L. K. KORHONEN, and E. KORHONEN Electrophoretic separation of carbonic anhydrase and naphthyl esterase activities *Ann Med exp Fenn* 1966 44 63-66
- HÄRKÖNEN, M. Carboxylic esterase oxidative enzymes and catecholamines in the superior cervical ganglion of the rat and the effect of pre and postganglionic nerve division *Acta physiol scand* 1964 Suppl 23.
- ICHIKAWA, A. Electron microscope study on secretion of the rat adenohypophysis *Acta anat nippon* 1959 34 460-482
- IKONEN, M. Ihmisen kiveksen välisolujen rakenteesta ja toiminnasta sikiöhaudella. Doctoral Thesis Univ of Helsinki 1965
- JIPÁSEK, I. E., Die Histotopochemie einiger Enzyme in der fetalen Adenohypophyse des Menschen. *Acta histochem (Jena)* 1963 10 42-49
- JOHNSTON, R. B. Thiolesterase activity of papain *J Biol Chem* 1956 221 1037-1046
- KIEF, H. Experimentelle Untersuchungen über die zelluläre Bildungsstätte des ACTH *Beitr path Anat* 1956 116 541-573
- KNIGGE, K. M. Cytology and hormone content of rat pituitary glands following adrenal ectomy *Proc Soc exp Biol (NY)* 1957 94 640-643
- KNIGGE, K. M. Cytology and growth hormone content of rat's pituitary gland following thyroidectomy and stress *Anat Rec* 1959 130 543-551
- KNUTSOV, F. On the cellular site of corticotrophin production *Acta endocr (Kbh)* 1966 52 305-318
- KOBAYASHI, H. and D. S. FARNER Cholinesterases in the hypothalamo-hypophysial neurosecretory system of the white crowned sparrow, *Zonotrichia leucophrys gambelii* *Z Zellforsch* 1964 63 965-973
- KOELLE, G. B. The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions *J Pharmacol exp Ther* 1951 103 153-171
- KOELLE, G. B. and C. N. GEESKY Localization of acetylcholinesterase in the neurohypophysis and its functional implications *Proc Soc exp Biol (NY)* 1961 106 605-608
- KOFFLER, D. and M. FOGEL Immunofluorescent localization of LH and FSH in the human adenohypophysis *Proc Soc exp Biol (NY)* 1964 115 1080-1082

- KOKKO, A., Histochemical and cytophotometric observations on esterases in the spinal ganglion of the rat *Acta physiol scand* 196, Suppl 261
- KRACHT, J., H J BREUSTEDT, and U HACHMEISTER, Immunhistologische Befunde mit Anti  $\beta$ -Corticotropin am Hypophysenvorderlappen der Ratte *Naturwissenschaften* 1963a 50 432—433
- KRACHT, J., U HACHMEISTER, K FISCHER, and H J BREUSTEDT, Immunfluoreszenz der ACTH Bildung tatten *Verh dtsch Ges Path* 1963b 49 304—326
- KUFOSUMI K and Y KOBAYASHI, Corticotrophs in the anterior pituitary glands of normal and adrenalectomized rats as revealed by electron microscopy *Endocrinology* 1966 78 745—753
- KÖNIG, P A, Histotopochemie von Enzymen im menschlichen Ovarium *Fortschr Gynäk Gynäk* 1965 23 77—203
- LACOUR, F., Recherches sur la relation entre les cellules hypophysaires à granulations orangées (cellules  $\alpha$  de Rameis) et les phénomènes de lactation *C R Soc Biol (Paris)* 1950 144 248—249
- LAGUNOFF, D and E P BENDITT Benzoylsalicylanilide ester substrates of proteolytic enzymes Kinetic and histochemical studies I Chymotrypsin *Biochemistry* 1964 3 1427—1431
- LEDUC E H and G B WISLOCKI The histochemical localization of acid and alkaline phosphatases, non specific esterase and succinic dehydrogenase in the structures comprising the hematoencephalic barrier of the rat *J comp Neurol* 1959 97 241—290
- LEZNOFF A J FISHMAN I GOODFRIEND F MCGARRY J BECK and B ROSE, Localization of fluorescent antibodies to human growth hormone in human anterior pituitary glands *Proc Soc exp Biol (NY)* 1960 104 230—235
- LEZNOFF A J FISHMAN M TALBOT, F E MCGARRY J C BECK and B ROSE The cytological localization of ACTH in the human pituitary *J clin Invest* 1962 41 1740—1754
- LOJDA Z, Cytotopochemie fermentu v krysí hypofýzy *Čs Morfol* 1960 8 148—151
- LOJDA Z and V SCHREIBER Cytotopochemická studie enzymu hypofýzy krysí *Čs Fy siol* 1960 9 377—378
- LOJDA Z and V SCHREIBER Activités enzymatiques de l'adenohypophyse du Rat dans diverses conditions expérimentales *J Physiol (Paris)* 1964 56 599—600
- MAILLARD M, Origine des grains de sécrétion dans les cellules de l'antéhypophyse embryonnaire du rat rôle de l'appareil de Golgi *J Microscopie* 1963 2 81—94
- MARKERT C L and P L HUYER The distribution of esterases in mouse tissues *J Histochem Cytochem* 1959 7 42—49
- MARKERT C L and P MÖLLER Multiple forms of enzymes tissue ontogenetic, and species specific patterns *Proc nat Acad Sci (NY)* 1959 45 753—763
- MARSHALL J M JR Localization of adrenocorticotrophic hormone by histochemical and immunochemical methods *J exp Med* 1951 94 21—30
- MARTIN C J and A E AXELFORD Proteolytic enzyme system of skin II Characterization of esterase activities *Biochim Biophys Acta (Amst)* 1957 26 490—501
- MCHAY D G E C ADAMS A T HERTIG and S DANZIGER Histochemical horizons in human embryos II 6 and 7 millimeters embryos — Streeter's horizon XIV *Anat Rec* 1951 106 433—463
- MEITES J and C W TURNER Studies concerning the induction and maintenance of lactation I The mechanism controlling the initiation of lactation at parturition *Pes Bull Missouri Agric Exp Sta No* 415 1949

- METZALS J, The innervation of the anterior pituitary gland in the cat In *Patho-physiologia Dienecephalica* Springer Verlag Wien 1959 149-159
- MEYER P K C BIDLLEH, and J C FINERT, Pituitary gonad interaction in immature female parabiotic rats *Endocrinology* 1946 39 23-31
- MIDDLEY A P Jr Immunofluorescent localization of human pituitary luteinizing hormone *Exp Cell Res* 1963 32 606-609
- MILLER, F Elektronenmikroskopisch histochemischer Nachweis von saurer Esterase in Linsenomen der Leber *Beitr path Anat* 1961 110 233-261
- MINARI O, and D H ZHILINSKY Use of KCN for tabilization of color in direct nesslerization of Kjeldahl digests *Anat Histochem* 1963 6 320-327
- MOFF T MAEDA and Y SUHIMIZU Electron microscopic histochemistry of cholinesterases in the rat brain *Histochemie* 1964 4 63-64
- MOFFIS C J O R, D S RUSSELL, F W LANGFEBE and C M MITCHELL The melanophore expanding and corticotrophic activity of human pituitary tissue *J Endocr* 1966 14 263-267
- MYERS D K A SCHOTTE H BOER, and H BORSJE BAKKER Studies on esterases and other lipid hydrolysing enzymes 3 Inhibition of the esterase of pancreas *Biochem J* 1965 61 521-529
- MYERS D K J W TOL and M H T DE JONGE, Studies on esterases 5 Substrate specificity of the esterases of some saprophytic microbacteria *Biochem J* 1967 65 23-239
- NACHLAS M M and A M SELIGMAN The histochemical demonstration of esterase *J natl Cancer Inst* 1949a 9 415-423
- NACHLAS M M and A M SELIGMAN The comparative distribution of esterase in the tissues of five mammals by a histochemical technique *Anat Rec* 1949b 105 617-695
- NEURATH H and G W SCHWEPT, The mode of action of the crystalline pancreatic proteolytic enzymes *Chem Rev* 1950 46 69-133
- NIEMI M M HARKONEN and M IKONEN A chemical and histochemical study on the significance of the non specific esterase activity in the adult rat testis *Endocrinology* 1966 (in press)
- NIEMI M and M IKONEN Cytochemistry of oxidative enzyme systems in the Leydig cells of the rat testis and their functional significance *Endocrinology* 1966 70 161-174
- NOVIKOFF A B E ESSNER L BIENFICA and P IACIOFANO Lysosomes and secretory granules electron microscopic examination of enzyme reaction product *J Histochem Cytochem* 1966 10 654
- NOVIKOFF A B E PODDER J RAY and E NOE Biochemical heterogeneity of the cytoplasmic particles isolated from rat liver homogenate *J Histochem Cytochem* 1966 14 27-46
- OPFSTEIN L and E DAVIS Disc electrophoresis Parts I and II Distillation In *diatrie* Rochester N Y 1966
- PAESI F J A S E DE JONGH and S CROSSBUTH The effect of a low dose age of testosterone on the ICSH and FSH contents of intact and gonadectomized male and female rats (With additional data on body weight and various organ weights) *Acta endoc (Kbh)* 1959 30 239-267
- PAESI F J A S E DE JONGH M J HOOGSTRA and A ENGELBERT The follicle stimulating hormone content of the hypophysis of the rat as influenced by gonadectomy and estrogen treatment *Acta endoc (Kbh)* 1960 19 49-60

- IAESI, F J A B E DE JONCH, and C H WILLEMS Testosterone and the pituitary ICSH content of male and female rats (With additional remarks on body growth) *Arch int Pharmacodyn* 1959 116 217-221
- PAGET G L and E ECCLESTON Simultaneous specific demonstration of thyrotroph gonadotroph and acidophil cells in the anterior hypophysis *Stain Technol* 1960 35 119-122
- IASETTO N Il sistema acetilcolina acetilcolinesterasi nell'ipofisi In *Pathophysiology Diencephalica* Springer Verlag Wien 1958 773-775
- IASTEELS J L, Étude expérimentale des cellules responsables de la sécrétion de prolactine chez les Mammifères In *Cytologie de l'Adenohypophyse* Colloq Int Centre Natl Rech Sci (Paris) 1963 128 137-148
- PASTEELS, J L and M HERLANT, Notions nouvelles sur la cytologie de l'antéhypophyse chez le rat *Z Zellforsch* 1962 56 20-39
- PEARSE A G E, The cytochemical demonstration of gonadotropic hormone in the human anterior hypophysis *J Path Bact* 1949 61 195-202
- PEARSE A G E, Differential stain for the human and animal anterior hypophysis *Stain Technol* 1950 25 95-102
- PEARSE A G E Observations on the localisation nature and chemical constitution of some components of the anterior hypophysis *J Path Bact* 1952 64 791-809
- PEARSE, A G E The esterase of the hypophysis and their functional significance *J Path Bact* 1956 72 471-487
- PEARSE, A G E Esterases of the hypothalamus and neurohypophysis and their functional significance In *Pathophysiology Diencephalica* Springer Verlag Wien 1959 329-330
- PEARSE A G E *Histochemistry Theoretical and Applied* Churchill, London 1961
- PEARSE A G E, and S VAN NOORDEN The histoenzymology of the human adenohypophysis In *Cytologie de l'Adenohypophyse* Colloq Int Centre Natl Rech Sci (Paris) 1963a 128 63-72
- PEARSE, A G E, and S VAN NOORDEN, The functional cytology of the human adenohypophysis *Canad med Ass J* 1963b 88 46-471
- PEPLER W J and A G E PEARSE The histochemistry of the esterases of rat brain with special reference to those of the hypothalamic nuclei *J Neurochem* 1957 1 193-202
- PFLAIDERER G and P G CELLIERS Isolierung einer Amino-peptidase aus Nierenpartikeln *Biochem Z* 1963 359 186-189
- PERSS F M R H POLTER and J CEBRA The isolation and properties of a proteolytic enzyme cathepsin D from bovine spleen *Biochem J* 1960 74 501-514
- PERVES H D Morphology of the hypophysis related to its function In *Sex and Internal Secretion* Vol 1 The Williams and Wilkins Company, Baltimore 1961 161-239
- PERVES H D and W E GRIESBACH Observations on the acidophil cell changes in the pituitary in thyroxine deficiency states I Acidophil degranulation in relation to goitrogenic agents and extrathyroidal thyroxine *Brit J exp Path* 1946 27 170-179
- PERVES H D and W E GRIESBACH The site of thyrotrophin and gonadotrophin production in the rat pituitary studied by McManus Hotchkiss staining for glycoprotein *Endocrinology* 1951a 49 244-261

- PURVES H D and W E GRIESBACH, Specific staining of the thyrotrophic cells of the rat pituitary by the Gomori stain *Endocrinology* 1951b 49 427—428
- PURVES H D, and W E GRIESBACH, The significance of the Gomori staining of the basophils of the rat pituitary *Endocrinology* 1951c 49 652—662
- PURVES, H D and W E GRIESBACH Functional differentiation in the acidophil cells and the gonadotrophic basophil cells of the rat pituitary *Proc Univ Otago med Sch* 1951 30 27
- PURVES, H D, and W E GRIESBACH, The site of follicle stimulating and luteinizing hormone production in the rat pituitary *Endocrinology* 1954 55 785—793
- PURVES H D, and W E GRIESBACH Changes in the gonadotrophs in the rat pituitary after gonadectomy *Endocrinology* 1955 56 374—386
- PURVES, H D, and W E GRIESBACH Changes in the basophil cells of the rat pituitary after thyroidectomy *J Endocr* 1956 13 365—375
- QUENUM, A and M HERLANT Etude des cellules corticotropes dans diverses conditions expérimentales chez le Rat de Gambie (*Cricetomys gambianus*) *C R Acad Sci (Paris)* 1964 258 1902—1904
- RACADOT J Mise en évidence des cellules corticotropes de l'adénohypophyse chez le Chat impubère traité par des inhibiteurs de la stéroïdogenèse *C R Acad Sci (Paris)* 1963a 257 1910—1913
- RACADOT J, Mise en évidence histochimique d'acide sialique dans l'adénohypophyse *C R Soc Biol (Paris)* 1963b 157 496—499
- RACADOT J L'origine cytologique de l'hormone corticotrope de l'hypophyse chez le Chat impubère *C R Soc Biol (Paris)* 1963c 157 1931—1934
- RACADOT J and M HERLANT L'action de l'amphénone sur l'hypophyse du Chat impubère *Ann Endocr (Paris)* 1960 21 823—830
- REECE H P and C W TURNER Experimentally altering galactin content of the rat pituitary *Proc Soc exp Biol (NY)* 1937 36 283—285
- RENNELS E G Localization of phospholipids in the rat hypophysis *Anat Rec* 1953 115 659—671
- RENNELS, E G, Two tinctorial types of gonadotrophic cells in the rat hypophysis *Z Zellforsch* 1957 45 464—471
- RENNELS E G Gonadotrophic cells of rat hypophysis In *Cytologie de l'Adénohypophyse Colloq Int Centre Natl Rech Sci. (Paris)* 1963 128 201—213
- RENNELS E G, Sialic acid concentrations in the pituitary glands of normal and castrated rats *Endocrinology* 1965 76 994—995
- RENNELS E G and J F HOOD Sialic acid concentrations in the pituitary glands of normal and ovariectomized rats *Science* 1964 144 416—417
- RIEKKINEN P J, and V K HORSU Studies in the salivary glands II Hydrolysis of chromogenic ester substrates *Ann Med exp Fenn* 1965 43 15—20
- RINKHAFT, J F and M G FARQUHAR The fine vascular organization of the anterior pituitary gland An electron microscopic study with histochemical correlations *Anat Rec* 1965 121 207—239
- ROBYN C Y BOSSAERT P O HUBINONT J L PASTRELS and M HERLANT Détection des cellules LH dans l'hypophyse humaine par une réaction d'immunofluorescence *C R Acad Sci (Paris)* 1964 259 1° 6—1° 8
- ROMEIS B Die Hypophyse In *Handbuch der mikroskopischen Anatomie des Menschen* Vol 6 Part 3 Julius Springer Berlin 1940



- ROSSI, F. G. PESAROTTO and E. PEALE. Ricerche istochimiche sugli enzimi idrolizzanti agli esterî degli acidi carbossilici con l'impiego di substrati naftolici su materiale umano in sviluppo *Monit zool ital* 1954 65 384-386
- ROSSI, F. G. PESAROTTO, and E. PEALE. Embryogénèse humaine et activité estérasique non spécifique *Ann Histochim* 1957 2 113-126
- RUMAE P. and N. C. J. J. LADIGES. Comparative immunochemical studies with antisera to sheep prolactin and bovine growth hormone on anterior pituitaries of ox, sheep, rats and mice *Z Zellforsch* 1963 67 575-593
- SANDBERG A. E. and E. C. KENNETH. Evidence on the cellular source of luteotrophin derived from a study of rat pituitary autografts *Z Zellforsch* 1959 49 263-274
- SANO M. Further studies on the theta cell of the mouse anterior pituitary as revealed by electron microscopy with special reference to the mode of secretion *J Cell Biol* 1962 15 85-91
- SEVERINGHAUS, A. E. Anterior hypophyseal cytology in relation to the reproductive hormone. In *Sex and Internal Secretions* The Williams and Wilkins Company, Baltimore 1939 1045-1087
- SHAW, C. P. F. N. SHYDEL, and R. E. TASHIAN. New genetically determined molecular form of erythrocyte esterase in man *Science* 1960 138 31-32
- SHIBASO, S. and A. L. TAPPEL. Distribution of esterases in rat liver *Arch Biochem Biophys* 1964 106 250-266
- SHIPPEY G. S. and F. BINKLEY. Amino acid esterase activities of swine kidney *J Biol Chem* 1958 230 699-705
- SHVITKA, T. K., and A. M. SALIGMAN. Role of esterase inhibition on localization of esterase and the simultaneous cytochemical demonstration of inhibitor sensitive and resistant enzymic species *J Histochem Cytochem* 1961 9 504-527
- SIFERSTEIN, E. R. Identification of the adrenocorticotrophin producing cells in the rat hypophysis by autoradiography *J Cell Biol* 1963 17 521-546
- SIFERSTEIN, E. R., and V. F. ALLISON. Fine structure of the cells responsible for secretion of adrenocorticotrophin in the adrenalectomized rat *Endocrinology* 1965 76 70-79
- SOBEL, H. J. Relationship of three lysosomal enzymes to the Golgi zone and secretory activity in the rat pituitary and thyroid glands *Anat Rec* 1962 143 380-397
- SOBEL, H. J. Cytochemical localization of ten oxidative enzymes of rat anterior pituitary and thyroid glands during various phases of secretory activity *J Endocr* 1964 29 1-7
- STEGEMANN H. Eine Mikrobestimmung von Amidstickstoff speziell in Proteinen *Hoppe Seylers Z physiol Chem* 1958 312 255-263
- STIGLIANI P. L. MAGGI and M. FANFANI. Ricerche sperimentale sulla genesi dell'ormone corticotropo. Effetti della somministrazione di granuli ipofisari eosinofili e basofili in un ratto ipofisectomizzato *Arch De Vecchi Anat pat* 1951 22 801-846
- SVENSMARK O. Enzymatic and molecular properties of cholinesterases in human liver *Acta physiol scand* 1963 69 38-399
- SVENSMARK O. Molecular properties of cholinesterases *Acta physiol scand* 1965 Suppl 245
- SWIFTENHAM K. The buffered performic acid alcian blue periodic acid Schiff method for the differentiation of basophils in the human and rat pituitary *J clin Path* 1960 13 256-260

- RÖDERHOLM, U. Histochemical localization of esterase phosphatases and tetrazolium reductases in the motor neurons of the spinal cord of the rat and the effect of nerve division *Acta physiol scand* 1965 Suppl 236
- TAPPAN D A M J JACOB and H M BOYDEN Carboxate anhydrous isozymes of neonatal and adult human and some animal erythrocytes *Ann NY Acad Sci* 1964 121 549-599
- TIXIER VIDAL A, Histophy siologie de l'adenohypophyse des Oiseaux In *Cytologie de l'adenohypophyse* Colloq Int Centre Natl Rech Sci Paris 1963 128 255-273
- TORACK R M, and R J BARNETT, Fine structural localization of cholinesterase activity in the rat brain stem *Exp Neurol* 1962 6 224-244
- TOSCHI G A biochemical study of brain microsomes *Exp Cell Res* 1969 16 232-234
- UNDERHAY, E, S J HOLT H BEALFAY and C DE DUVE Intracellular localization of esterase in rat liver *J biophys biochem Cytol* 1966 2 635-637
- VANHA PERTTULA, T P J Studies on the adenohypophyseal esterases In *Proc 2nd Int Cong Histo and Cytochem* Springer Verlag Berlin Göttingen, Heidelberg 1964 143-144
- VANHA PERTTULA T P J, and V K HORSU Esterolytic and proteolytic enzymes of the rat adenohypophysis I Studies with homogenate and its fractions *Histochemia* 1965a 4 319-338
- VANHA PERTTULA T P J and V K HORSU Esterolytic and proteolytic enzymes of the adenohypophysis II Chromatographic fractionation and characterization of enzyme activities hydrolysing leucyl  $\beta$  naphthylamide *Ann Med exp Fenn* 1965b 43 39-39
- VANHA PERTTULA T V K HORSU and G G GLENNER Enzymes in hog kidney hydrolysing amino acid naphthylamides *J Histochem Cytochem* 1966 14 314-325
- VANHA PERTTULA T V K HORSU, V SOVINEN and G G GLENNER Cathepsin B related to some histochemical substrates *Histochemia* 1966 5 170-181
- VAN OORDT P G W J, Nomenclature of the hormone producing cells in the adenohypophysis A report of the International Committee for Nomenclature of the Adenohypophysis *Gen comp Endocr* 1965 5 131-134
- VAN REES G I Influence of steroid ex hormones on the FSH release by rat hypophyses *in vitro Acta endocr (Kbh)* 1961 36 495-497
- VERNE J La cytochimie d esterases chez les mammifères *Ann Biol* 1954 30 149-143
- VERNE J and S HÉBERT Etude cytochimique de l'activité esterase dans les glandes endocrines hormonogènes *Ann Endocr (Paris)* 195 18 659-663
- WACHSTEIN M and E MEISSEL Histochemistry of thioacetic acid esterase in relation to De Duve's lysosomes *J Histochem Cytochem* 1960 8 31-319
- WACHSTEIN M E MEISSEL and C PALCOV, Histochemistry of thioacetic acid esterase a comparison with nonspecific esterase with special regard to the effect of fixatives and inhibitors on intracellular localization *J Histochem Cytochem* 1961 9 325-339
- WATTIAUX R and C DE DUVE Tissue fractionation studies VII Release of bound hydrolases by means of Triton X 100 *Biochem J* 1966 63 606-608
- WEBB F C The nomenclature of multiple enzyme forms *Enymol biol clin* 1965 5 124-125
- WEISS M The release of pituitary secretion in the platyfish *Xiphophorus maculatus* (Guenther) *Z Zellforsch* 1965 68 783-794

- WIGGANS D E M WINITZ, and J S FRUTON, Action of cathepsin B on dipeptide esters *Falc J Biol Med* 1954 27 11—19
- WACHTLE K, and A G E PEARSE, The histochemical demonstration of five lysosomal enzymes in the pars distalis of the amphibian pituitary *Z Zellforsch* 1966 69 326—333
- YOSHIMURA, F and K. HARUMIYA, Electron microscopy of the anterior lobe of pituitary in normal and castrated rats *Endocr jap* 1964 12 119—152
- YOSHIMURA F K HARUMIYA R ITO, and N SUZUKI, A doubt for the classification of thyrotroph and gonadotroph in the anterior lobe of rat pituitary *Endocr jap* 1964 11 101—111
- ZECHNER I T L W DAVISON T B KELLER and S C LIVINGOOD, The pituitary in experimental cretinism structural changes in the pituitaries of thyroidectomized rats *Amer J med Sci* 1935 190 145—157





ACTA PHYSIOLOGICA SCANDINAVICA

Vol. 69 Supplementum 284

STUDIES ON  
VASCULAR PERMEABILITY IN  
PERIPHERAL NERVES

2 Distribution of circulating fluorescent serum albumin in  
rat sciatic nerve after local injection of 5 hydroxytryptamine,  
histamine and Compound 48/80

By

Yngve Olsson

GÖTEBORG 1966



From the Neuropathological Laboratory Department of Pathology I  
University of Göteborg Sweden

STUDIES ON  
VASCULAR PERMEABILITY IN  
PERIPHERAL NERVES

2 Distribution of circulating fluorescent serum albumin in  
rat sciatic nerve after local injection of 5 hydroxytryptamine  
histamine and Compound 48/80

By

Yngve Olsson



ORSTADIUS BOKTRYCKERI AKTIEBOLAG  
GUTEBORG 1966

The vascular architecture of peripheral nerves has been described by several authors (Adams 1942 Sunderland 1945 Richards 1951, Waksman 1961 Edshage 1964) In the epineurium and the perineurium there are vascular networks which communicate with a plexus of small anastomosing blood vessels in the endoneurium of the nerves

There seems to be a difference in permeability between the epineurial and the endoneurial blood vessels of rat sciatic nerves under physiological conditions (Olsson 1966 a) After intravenous injection of fluorescent labelled serum albumin fluorescence appeared both in the lumen and in the walls of epineurial blood vessels In addition a diffuse faint extra vascular fluorescence was observed which extended to the innermost parts of the epineurium and the perineurium In the endoneurium however, fluorescence was only observed in the lumen of blood vessels No fluorescence was detected in or outside the walls of these blood vessels

Traumatic lesions of rat sciatic nerves are accompanied by a rapid and marked increase in the permeability of both endoneurial and epineurial blood vessels for fluorescent labelled serum albumin (Olsson 1966 a) The most marked exudation is observed at the site of the lesion during the initial hours after the trauma Simultaneously, the exsuded fluorescent albumin spreads chiefly into the distal endoneurium Corresponding observations have recently been made by Mellick (1966) who found that physical trauma to chicken sciatic nerves is associated with a rapid increase in the wet weight most pronounced in the segments adjacent to and distal of the lesion Quantitative determinations of intravenously injected  $J^{131}$  labelled serum albumin revealed a marked increase of labelled albumin in the corresponding segments

Increased vascular permeability for serum proteins and formation of protein rich exudate are cardinal features of the acute inflammatory process (Spector & Willoughby 1963 1964 1965 Majno 1964, Wilhelm 1962 1965) It is generally held that this increased vascular permeability can be induced by direct damage to vascular walls and by liberated endogenous chemical mediators

Of various mediators histamine and 5 hydroxytryptamine (5 HT serotonin) have the capacity to induce a rapid transient increase in the permeability of blood vessels in several tissues (Spector & Willoughby

1963 1964, 1965, Majno 1964 Wilhelm 1962 1965) The central nervous system appears however, to be an exception Thus Broman and Lindberg-Broman (1945) reported that cerebral blood vessels entirely resisted the action of histamine, even in large doses The effect of histamine and 5 hydroxytryptamine on blood vessels in peripheral nerves does not seem to have been described previously

Unlike the nerve fibres of the brain those of the peripheral nerves are separated by connective tissue having a substantial extracellular space (Thomas 1963, Gamble 1964, Waggner Bunn & Beggs 1965) Mast cells are normally present in the connective tissue of the nerves both in the epineurium the perineurium and the endoneurium (Enerback Olsson & Sourander 1965) Those cells most probably store the major part of the histamine and 5 hydroxytryptamine normally contained in rat peripheral nerves (Kwiatkowski 1943 Torp 1961, Olsson 1965 Anden & Olsson 1966)

Numerous authors have observed that the onset of the acute inflammatory process in several tissues of the rat is associated with degranulation of mast cells (Maximow 1904 Ernst 1926 McGovern 1957, Gustavsson & Cronberg 1957 Spector & Willoughby 1959 Sheldon & Bauer 1960) These cells are highly responsive and fragile A large number of stimuli e.g. mechanical trauma heat cold ionizing radiation and several chemical and biological agents have the capacity to induce degranulation of mast cells and liberation of histamine and 5 hydroxytryptamine (Moran, Uvnas & Westerholm 1962 Uvnas 1964) One of the most potent mast cell degranulating agents is Compound 48/80 a condensation product between paramethoxyphenethylmethylamin and formaldehyde (Baltzy Buck, deBeer & Webb 1949) Several investigators have also observed that local injection of this compound into various tissues of the rat is associated with degranulation of mast cells and increased vascular permeability leading to the formation of oedema (Rowley & Benditt 1956 Sparrow & Wilhelm 1957 Gotzy & Kato 1964 Rowley 1964)

The aim of the present study was to investigate the effect of the mast cell amines histamine and 5 hydroxytryptamine on the vascular permeability in peripheral nerves In addition the effect of the histamine liberator Compound 48/80 was studied in the same way

## MATERIALS AND METHODS

Vascular permeability changes was demonstrated by fluorescence microscopic tracing of intravenously injected serum albumin tagged with Evans blue according to Steinwall and Klatzo (1965). Histamine 5 hydroxytryptamine and Compound 48/80 were injected into the endoneurium or applied to the surface of the epineurium of rat sciatic nerves. Some of the animals were pretreated with a histamine antagonist mepyramine maleate others with a serotonin antagonist methysergide bimaleinate.

Previous studies have revealed that mepyramine maleate has a strong antihistaminic action (Spector & Willoughby 1965, Swanes 1965). It has also been shown that methysergide bimaleinate has a marked antagonistic effect on the serotonin induced oedema in the skin of rats (Fanchamps Doepfner, Weidmann & Cerletti 1960) and mice (Swanes 1965).

### *Animals*

164 Sprague Dawley rats of both sexes were used weighing between 250 and 350 g. They were kept on a diet of commercial rat pellets and water ad libitum. The injections were performed under ether anaesthesia. At suitable times, varying between five min and 24 hrs after the injections rats were killed by bleeding from a cut in the heart.

### **I Microinjections into the endoneurium of sciatic nerves**

Injections into the endoneurium of sciatic nerves were performed as previously described (Olsson 1966 b). The nerves were exposed high up in the thigh. A very fine glass capillary connected via a short plastic tube to a micrometer syringe<sup>1)</sup> was used for the injections. The glass capillary was inserted distally into the nerves. Both sciatic nerves were used in each rat with less than two min delay between the injections.

Groups of three to five rats received Evans blue albumin (EBA) either before or after injection of the permeability changing agents as stated below.

### *Controls*

Groups of rats were injected with EBA immediately followed by a single endoneurial injection of 0.002, 0.004 or 0.006 ml Ringer solution

<sup>1)</sup> Agla Micrometer Syringe Barroughs Wellcome & Co. England

<sup>2)</sup> Evans blue albumin abbreviated EBA (Klatzo & Steinwall 1965)

respectively. They were killed after 15 min. Groups of rats injected with 0.002 ml Ringer solution were also killed 2 and 6 hrs after the injection.

In subsequent experiments the permeability changing agents were dissolved in Ringer solution. For microinjections into the endoneurium a volume of 0.002 ml was used in all the experiments.

### *5-hydroxytryptamine*

Groups of rats were injected with EBA immediately prior to the local injection of 1  $\mu$ g 5 hydroxytryptamine<sup>3)</sup>. They were killed after 15 min and 2 hrs respectively.

The duration of the permeability increasing capacity of 5 hydroxytryptamine was studied in one group of rats by the injection of 1  $\mu$ g 5 hydroxytryptamine into the nerves followed 15 min later by EBA. The animals were killed 30 min after the last injection.

Two groups received subcutaneously 0.1 mg methysergide<sup>4)</sup> per 100 g body weight, 30 min later EBA immediately followed by the injection of 1  $\mu$ g 5 hydroxytryptamine. They were killed 30 min after the last injection.

### *Histamine*

Groups of rats were injected with EBA immediately prior to the local injection of 1  $\mu$ g histamine<sup>5)</sup>. They were killed after 15 min and 2 hrs respectively.

Other groups of rats were injected with EBA immediately prior to the local injection of 10  $\mu$ g histamine. They were killed after 15 min and 2 hrs respectively.

The duration of the permeability increasing effect of histamine was studied in one group of rats injected with 10  $\mu$ g histamine into the nerves followed 15 min later with EBA. The rats were killed 30 min after the last injection.

Two groups received subcutaneously 0.1 mg mepyramine<sup>6)</sup> per 100 g body weight, 30 min later EBA immediately followed by the injection of 10  $\mu$ g histamine. The rats were killed 30 min after the last injection.

<sup>3)</sup> 5 hydroxytryptamine serotonin creatinin sulphate obtained from Sigma Chemical Co. St. Louis, U.S.A.

<sup>4)</sup> Methysergide bimaleinate 1 methyl D lysergic acid butanolamide Deseril® kindly supplied by Sandoz Ltd. Basel, Switzerland.

<sup>5)</sup> Histamine dihydrochloride obtained from Fluka AG Chemische Fabrik, Switzerland.

<sup>6)</sup> Mepyramine maleate, Antihisan® kindly supplied by May & Baker Ltd. Dagenham, England.

### *Compound 48/80*

Groups of rats were injected with EBA immediately followed by the local injection of 10  $\mu$ g Compound 48/80<sup>†</sup> They were killed 15 min and 2 hrs after the last injection

The duration of the permeability increasing capacity of Compound 48/80 was studied in groups of rats locally injected with 10  $\mu$ g Compound 48/80 followed 15 min and 2 hrs later respectively by the injection of EBA They were killed 30 min later

Groups of rats received both methysergide and mepyramine subcutaneously (0.1 mg per 100 g body weight) 30 min later EBA immediately followed by the local injection of 10  $\mu$ g Compound 48/80 They were killed 15 min and 2 hrs later respectively

## **II Local application to the epineurium of exposed sciatic nerves**

The sciatic nerves were exposed by making small incisions high up in the thigh Ringer solution was first applied around the right sciatic nerve immediately followed by injection of the permeability changing agents dissolved in Ringer solution around the left nerve The dosages were the same as those used by Rowley and Benditt (1956) and Rowley (1964) in studies on rat paws i.e. 0.1 ml 5-hydroxytryptamine (2  $\mu$ g/ml) histamine (400  $\mu$ g/ml) and Compound 48/80 (10  $\mu$ g/ml)

The experimental groups corresponded to those used in the experiments with microinjections into the nerves Groups of rats were injected with EBA immediately followed by the local injections around the nerves They were killed after 5 min and 2 hrs respectively The duration of the permeability changing capacity was studied on other groups of rats which received local injections first, followed 15 min later by the injection of EBA These rats were killed 30 min later Other groups again were pre-treated with methysergide and mepyramine singly or in combination 30 min before the injections of EBA and the permeability changing agents They were killed either 15 min or 2 hrs later

### *Demonstration of vascular permeability*

The technique for demonstrating vascular permeability has been described in detail elsewhere (Olsson 1966a) Briefly each rat received intravenously 1 ml per 100 g body weight of a solution containing Evans

<sup>†</sup> Compound 48/80 a condensation product between paramethoxyphenethylmethylamine and formaldehyde kindly supplied by the Wellcome Research Laboratories Beckenham Kent England

blue<sup>8)</sup> in 5 % bovine albumin <sup>9)</sup> The sciatic nerves were fixed in 5 % formalin for 24 hrs Frozen longitudinal sections 10  $\mu$  thick, from various levels of the nerves were mounted in 50 % aqueous glycerin and immediately studied in a fluorescence microscope (equipped with a dark field condensor and light from an OSRAM HBO 200 W high pressure mercury lamp) The light was filtered through a Schott BG 12/3 mm filter In the tubes the emitted light was filtered through a combination of a Schott OG 4 and a GG 4 filter

The technique used here is based on the recent finding that EBA emits a strong red fluorescent light which permits detailed tracing of this substance in thin frozen sections (Steinwall & Klatzo 1965 Klatzo & Steinwall 1965)

---

<sup>8)</sup> Evans blau E Merck A G Darmstadt Germany

<sup>9)</sup> Nutritional Biochemicals Corporation Cleveland Ohio U.S.A

## RESULTS

### I Microinjections into the endoneurium of sciatic nerves

#### *Controls*

The injection of 0.002 ml Ringer solution did not appreciably disturb the distribution of fluorescent albumin (EBA) in the endoneurium compared with the distribution in normal nerves as described in a previous paper (Olsson 1966 a). All rats displayed only slight local changes at the site of the injection with small droplets of red fluorescent products appearing in the walls of a few endoneurial blood vessels (Fig. 1). The rest of the endoneurium appeared normal. Injections of 0.004 and 0.006 ml Ringer solution produced more pronounced exudation at the site of the injection with a diffuse faint extravascular fluorescence around the endoneurial blood vessels.

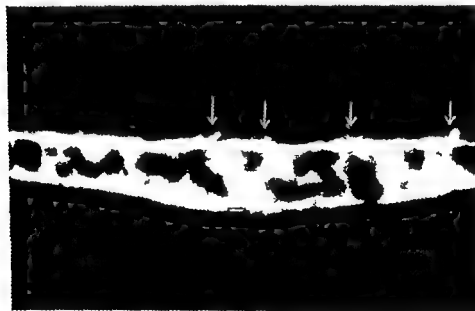


Fig. 1 Endoneurial injection of Ringer solution (control) into rat sciatic nerve preceded by intravenous injection of Evans blue albumin (EBA). Fluorescence in the lumen of an endoneurial blood vessel and a few droplets in the vascular walls (arrows).



### *5-hydroxytryptamine*

Local injection of 5 hydroxytryptamine preceded by intravenous injection of EBA resulted in a marked exudation of fluorescent material into the endoneurium of all rats. At the site of the injection an indistinct limited zone with a bright red diffuse fluorescence was observed between the nerve fibres (Fig 2). The amount of extravascular fluorescent material was most pronounced at the site of the injection. In parts remote from the injection site, a meshwork of fluorescent material appeared in the vascular walls and in addition there were multiple small fluorescent droplets just outside the endoneurial blood vessels (Fig 3). The extension of the exudated material did not differ between rats studied 15 min and 2 hrs after the injection.

When EBA was injected 15 min after the local injection of 5 hydroxytryptamine there was only a faint fluorescence in and just outside vascular walls at the site of the injection while the other parts of the nerves appeared normal (Fig 4).

Rats treated with methysergide before the local injection of 5-hydroxytryptamine displayed fluorescence in the lumen of endoneurial blood vessels and small fluorescent droplets in the walls of endoneurial blood vessels at the site of the injection (Fig 5).



Fig 2 Microinjection of 5 hydroxytryptamine preceded by intravenous injection of EBA. Vast intense fluorescence between the nerve fibres in the endoneurium.

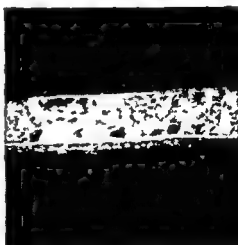


Fig 3 Leaking blood vessel in the endoneurium remote from the site of injection of 5 hydroxytryptamine.



Fig 4 Intravenous injection of EBA 15 min after local injection of 5 hydroxytryptamine Endoneurial blood vessel with normal appearance

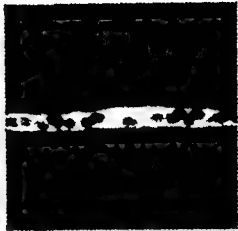


Fig 5 Pretreatment with methysergide before injections of EBA and 5 hydroxytryptamine Endoneurial blood vessel with normal appearance

Thus compared with local injections of Ringer solution the injections of 5 hydroxytryptamine induced a markedly increased permeability of endoneurial blood vessels. The increased permeability was of early onset and brief duration and could be inhibited by pretreatment of the rats with the 5 hydroxytryptamine-antagonist methysergide bimalenate.

### *Histamine*

Injections of 1  $\mu$ g histamine resulted in a discrete leakage of fluorescent material around endoneurial blood vessels at the site of the injection in twelve nerves. The remaining four nerves displayed the same distribution of fluorescent material as the controls.

The injection of 10  $\mu$ g histamine however resulted in a marked endoneurial exudation of fluorescent products in all the nerves. Fluorescence appeared not only the injection site but also around scattered endoneurial blood vessels along the whole sciatic nerve (Fig 6).

When EBA was injected 15 min after the local injection of 10  $\mu$ g histamine there was only a slight local exudation in two nerves while the other four appeared entirely normal.

Rats treated with mepyramine maleate before the local injection of 10  $\mu$ g histamine showed only a slight local exudation of fluorescent products at the site of the injection in all the nerves.



Fig 6 Microinjection of histamine into the endoneurium preceded by intravenous injection of EBA. Intense fluorescence in and outside the walls of an endoneurial blood vessel



Fig 7 15 min after endoneurial injection of Compound 48/80 preceded by intravenous injection of EBA. Fluorescence in the walls of an endoneurial blood vessel

Thus as for 5 hydroxytryptamine the permeability increasing capacity of histamine on endoneurial blood vessels was of early onset and brief duration. This effect was abolished by pretreatment of the rats with the histamine antagonist, mepyramine maleate. The different response between 5 hydroxytryptamine and histamine when the same dose was injected might reflect different permeability increasing activities of the two agents. Previously, the permeability increasing activity of 5 hydroxytryptamine has been found to be more powerful than that of histamine on blood vessels of the skin and the muscle of the rat (Rowley & Benditt 1956; Mijno & Palade 1961; Rowley 1964).

#### *Compound 48/80*

EBA administered to rats before the local injection of Compound 48/80 displayed intense red fluorescence 15 min later at the site of the injection both in and outside the walls of endoneurial blood vessels. There was also an extravasation around several blood vessels all along the nerve (Fig 7). Two hours after the injections a diffuse extravascular red fluorescence was observed in the endoneurium of all injected nerves (Fig 8). The most intense fluorescence appeared between nerve fibres especially in the vicinity of endoneurial blood vessels.



Fig 8 2 hrs after endoneurial injection of Compound 48/80 preceded by intravenous injection of EBA Note the diffuse rich extravascular fluorescence in the endoneurium

Rats injected with EBA 15 min and 2 hrs after the local injection of Compound 48/80 displayed fluorescence in and just outside scattered endoneurial vessels all along the nerve. In all cases the amount of extravascular fluorescent material was markedly less than in the preceding groups (Fig 9).

The combined treatment with methysergide and mepyramine before the local injection of Compound 48/80 resulted 15 min later in a weak to moderate diffuse extravascular fluorescence at the site of the injection and a patchy occurrence of small fluorescent droplets in and just outside the walls of endoneurial blood vessels in the other parts of the nerves (Fig 10). 2 hrs after the local injection a diffuse faint extravascular fluorescence was observed throughout the nerves.

Thus the local injections of Compound 48/80 were associated with a markedly increased permeability of endoneurial blood vessels with a rapid onset but which in contrast to the injections of either 5 hydroxytryptamine or histamine still persisted 2 hrs after the injection. Pretreatment of the rats with the 5 hydroxytryptamine antagonist methysergide and the histamine antagonist mepyramine markedly reduced but did not entirely inhibit the increased permeability.



Fig 9 Intravenous injection of EBA 2 hrs after local injection of Compound 48/80. Fluorescence in the walls of an endoneurial blood vessel



Fig 10 Pretreatment with methysergide and mepyramine before injection of EBA and Compound 48/80. The rat was killed 15 min later. Fluorescence in the walls of an endoneurial blood vessel

## II Local application to the epineurium of exposed sciatic nerves

When EBA was injected prior to the local application of either histamine 5 hydroxytryptamine or Compound 48/80, a diffuse intense extravascular fluorescence was observed after only 5 min (Fig 11)

The fluorescent products extended to the innermost parts of the epineurium and perineurium leaving the underlying parts of the endoneurium devoid of extravascular fluorescence. No difference in fluorescence intensity or extent of the fluorescent material could be detected between rats injected with histamine 5 hydroxytryptamine or Compound 48/80. On the control side of these rats numerous small fluorescent droplets and a faint diffuse extravascular fluorescence were observed in and outside the walls of epineurial and perineurial blood vessels.

15 min and 2 hrs after the local application of the permeability changing agents fluorescence in the epineurium and the perineurium was more intense than in the preceding group. No difference could be observed between the experimental nerves of rats studied 15 min and 2 hrs after the local injections. The control nerves of these rats displayed a diffuse faint extravascular fluorescence in the epineurium and the perineurium. The intensity of the fluorescent light was most marked in rats studied 2 hrs



a



b



c



d

Fig. 11 Intense diffuse fluorescence in the epineurium and the perineurium 5 min after local application of the permeability changing agents to the exposed sciatic nerve. Intravenous injection of EBA prior to the local application. Arrows mark the border between perineurium and endoneurium.

- a Histamine
- b 5-hydroxytryptamine
- c Compound 48/80
- d Control (local application of Ringer solution)

after the local application of Ringer solution. When EBA was injected 15 min after the local injection of permeability changing agents, no difference

could be detected between experimental and control nerves in the intensity or the extent of the extravascular fluorescence

Pretreatment of rats with methysergide eliminated the difference between experimental and control side after the local injection of 5-hydroxytryptamine and Ringer solution respectively. In a similar manner, mepyramine completely countered the difference observed after the injection of histamine and Ringer solution. After the combined pretreatment of rats with methysergide and mepyramine of rats subjected to local injections of Compound 48/80 and Ringer solution, no difference could be detected in the fluorescent microscopic appearance of the epineurium and the perineurium in the two nerves.

## DISCUSSION

The capacity of 5 hydroxytryptamine to heighten vascular permeability has previously been investigated after local injection into skin (Rowley & Benditt 1956 Parrat & West 1957 Rowley 1964) or cremaster muscle of the rat (Majno & Palade 1961 Majno Palade & Schoefl 1961). The permeability increasing effect of this amine is generally thought to be of rapid onset and brief duration (Spector & Willoughby 1963 1964 1965). Studies on whole specimens of the cremaster muscle of rats using circularizing carbon or mercuric sulfide for demonstration of vascular permeability, revealed that the part of the vascular tree affected by local injections of 5 hydroxytryptamine tended to be the venules rather than the capillaries (Majno Palade & Schoefl 1961). The detailed mechanism by which 5 hydroxytryptamine induces an increased permeability of blood vessels is not fully understood and the published results somewhat contradictory. Thus recent electronmicroscopic studies have demonstrated that numerous leaks develop around the junctions between adjacent endothelial cells (Majno & Palade 1961). In contrast Alksne (1959) in a study on the effect of histamine on the passage of colloidal mercuric sulfide particles across dermal capillary walls considered that the passage took place across the endothelial cytoplasm rather than through intercellular spaces. Both groups of investigators considered that the increased permeability was induced by the direct action of the amine on the endothelial cells. Rowley (1964) however demonstrated that the increased permeability might in part be secondary to a constriction of veins through the action of 5 hydroxytryptamine.

The present study shows that injections of 5 hydroxytryptamine into rat sciatic nerves are accompanied by the appearance of the tracer, EBA in and around vascular walls for a long distance along the endoneurium while control injections of Ringer solution only elicited slight local changes. The exudation of the tracer accompanying the local injection of 5 hydroxytryptamine was of brief duration and could be markedly reduced by pretreatment of the rats with the serotonin antagonist methysergide bitartrate.

The findings were similar in the epineurium after the local application of 5 hydroxytryptamine to the surface of exposed sciatic nerves. These results strongly suggest that both endoneurial and epineurial blood vessels



## SUMMARY

The effect of 5 hydroxytryptamine histamine and Compound 48/80 on the permeability of endoneurial and epineurial blood vessels in rat sciatic nerves was studied. The agents were microinjected into the endoneurium or applied to the epineurium of exposed nerves. Vascular permeability was demonstrated by fluorescent microscopic tracing of intravenously injected serum albumin labelled with Evans blue according to Steinwall and Klatzo (1965). The main findings were as follows:

1. Injections of 5 hydroxytryptamine and histamine were associated with a marked increased permeability of endoneurial blood vessels of rapid onset and brief duration. This effect was inhibited by pretreatment with a 5 hydroxytryptamine antagonist (methysergide bimalenate) and a histamine antagonist (mepyramine maleate) respectively.

2. Injections of Compound 48/80 were associated with a markedly increased permeability of endoneurial blood vessels with a rapid onset and lasting at least 2 hrs after the injection. Pretreatment of rats with mepyramine and methysergide in combination markedly reduced but did not entirely inhibit the increased permeability.

3. Applications of 5 hydroxytryptamine histamine and Compound 48/80 to the epineurium of exposed sciatic nerves were associated with a markedly increased permeability of epineurial blood vessels of rapid onset and brief duration. Pretreatment of rats with mepyramine completely eliminated the action of histamine, methysergide the action of 5 hydroxytryptamine and mepyramine and methysergide combined the effect of Compound 48/80.

Thus the mast cell amines histamine and 5 hydroxytryptamine are capable of inducing increased permeability of blood vessels in rat peripheral nerves. The permeability increasing capacity of Compound 48/80 seems to be mainly mediated by biogenic amines released from mast cells in the nerves.

## REFERENCES

- ADAMS W E The blood supply of nerves 1 Historical review *J Anat (Lond)* 6 323—341 1942
- ALASNE J F The passage of colloidal particles across the dermal capillary wall under the influence of histamine *Quart J Exp Physiol* 44 51—66 (1959)
- ANDÉN N F & OLSSON Y 5-hydroxytryptamine as normal and sectioned sciatic nerve of the rat To be published
- BALZLY R BLICK J S DE BEER E J & WEBB T J A family of long acting depressors *J Amer Chem Soc* 71 1301—1305 1949
- BROMAN T & LINDBERG BROMAN A M An experimental study of disorders in the permeability of the cerebral vessels (the blood brain barrier) produced by chemical and physico-chemical agents *Acta Physiol Scand* 10 102—125 1945
- DALE H H & LAIDLAW P P The physiological action of  $\beta$ -aminazolyethylamine *J Physiol (Lond)* 41 318—344 1910
- — Further observations on the action of  $\beta$ -aminazolyethylamine *J Physiol (Lond)* 43 187—195 1911
- — Histamine shock *J Physiol (Lond)* 52 355—390 1918
- EDSHAGE S Peripheral nerve suture A technique for improved intraneural topography and evaluation of some suture materials *Acta Chir Scand Suppl* 331 1964
- ENERBÄCK L OLSSON Y & SOLRÄNDER P Mast cells in normal and sectioned peripheral nerve *Z Zellforsch* 67 596—608 1965
- ERNST T Über die ersten Stunden der Entzündung *Beitr path Anat* 73 249—258 1926
- FANCHAMP A DOEPFNER W WEIDMANN H & CERLETTI A Pharmakologische Charakterisierung von Deseril® einem Serotonin Antagonisten *Schweiz Med Wochenschr* 90 1040—1046 1960
- GAMBLE H J Comparative electron microscopic observations on the connective tissues of a peripheral nerve and a spinal nerve root in the rat *J Anat (Lond)* 98 17—25 1964
- GLSTAVSSON B E & CRONBERG S Comparison of the effects of compound 48/80 protamine and turpentine oil on mast cell degranulation *Acta Rheumat Scand* 3 189—202 1957
- GOREY B & KATO L Effects of histamine serotonin and catecholamine depletors on the capillaries in the rat skin *Arch int Pharmacodyn* 148 243—254 1964
- KLATZO I & STENWALL O Observation on cerebrospinal fluid pathways and behaviour of the bloodbrain barrier in sharks *Acta neuropath (Berl)* 5 161—175 1965
- KWIATKOWSKI H Histamine in nervous tissue *J Physiol (Lond)* 107 32—41 1943
- LEWIS T The blood vessels of the human skin and their responses *Lond in Shaw & Sons Ltd* 1927
- MAJNO G Mechanisms of abnormal vascular permeability in acute inflammation In *Injury inflammation and immunity* edit by L Thomas J W Uhr and L Grant pp 58—93 Baltimore The Williams & Wilkins Co 1964
- & PALADE G E Studies on inflammation I The effect of histamine and serotonin on vascular permeability An electron microscopic study *J Biophys Biochem Cytol* 11 571—606 1961
- — & SCHÖEFL GUTTA, I Studies on inflammation II The site of action of histamine and serotonin along the vascular tree A topographic study *J Biophys Biochem Cytol* 11 607—616 1961
- MAYNARD A Über entzündliche Bindegewebsneubildung bei der weissen Ratte und die dabei auftretenden Veränderungen der Mastzellen und Fettzellen *Beitr path Anat* 35 93—126 1904
- MCGOVERA V J The mechanism of inflammation *J Path Bact* 71 99—106 1957
- MELLICK R S Thesis on blood vessel permeability in peripheral nerve following trauma and during Wallerian degeneration London University 1966
- MORAN N C UPPAS B and WESTERHOLM BARBRO Release of 5 hydroxytryptamine and histamine from rat mast cells *Acta Physiol Scand* 56 26—41 1962

- OLSSON Y Storage of monoamines in mast cells of normal and sectioned peripheral nerve  
Z Zellforsch 68 255—265 1965
- Studies on vascular permeability in peripheral nerves 1 Distribution of circulating fluorescent serum albumin in normal crushed and sectioned rat sciatic nerve Acta Neuropath (Berl) in press 1966a
- The effect of the histamine liberator Compound 48/80 on mast cells in normal peripheral nerves Acta Path Microbiol Scand in press 1966b
- PARRAT J R & WEST G B Inhibition by various substances of oedema formation in the hindpaw of the rat induced by 5 hydroxytryptamine histamine dextran eggwhite and compound 48/80 Brit J Pharmacol 13 65—00 1958
- RICHARDS R L Ischemic lesions of peripheral nerves A review J Neurol Neurosurg Psychiat 14 76—87 1951
- ROWLEY D A Venous constriction as the cause of increased vascular permeability produced by 5 hydroxytryptamine histamine bradykinin and 48/80 in the rat Brit J Exp Path 44 56—67 1964
- & BENDITT E P 5 hydroxytryptamine and histamine as mediators of the vascular injury produced by agents which damage mast cells in rats J Exp Med 103 399—411 1956
- SHIELDON W H & BAUER H Tissue mast cells and acute inflammation in experimental cutaneous mucormycosis of normal 48/80 treated and diabetic rats J Exp Med 112 1069—1083 1960
- SPARROW F M & WILHELM D L Species differences in susceptibility to capillary permeability factors Histamine 5 hydroxytryptamine and compound 48/80 J Physiol (Lond) 137 51—65 1957
- SPECTOR W G & WILLOUGHBY P A The demonstration of the role of mediators in turpentine pleurisy in rats by experimental suppression of the inflammatory changes J Path Bact 77 1—17 1959
- — The inflammatory response Bact Rev 27 117—154 1963
- — Vasoactive amines in acute inflammation Trans N Y Acad Sci 116 839—845 1964
- — Chemical mediators II In The inflammatory response pp 427—448 Ed by B W Zweifach L Grant and R T McCluskey New York and London Academic Press 1965
- STEINWALL O & KLATZO I Double tracer methods in studies on bloodbrain barrier dysfunction and brain edema Proc 17th Congr Scand Neurol Goteborg 1964 Acta neurol Scand 41 suppl 13 R 31/1—R 31/5 1965
- SUNDERLAND S Blood supply of the nerves of the upper limb in man Arch Neurol Psychiat (Chic) 53 91—115 1945
- SWANES K Studies in hypothermia 5 The role of reduced release of chemical mediators in turpentine inflammation in hypothermic mice Acta Path Microbiol Scand 65 13—23 1965
- THOMAS P K The connective tissue of peripheral nerve An electron microscopic study J Anat (Lond) 97 35—44 1963
- TORP A Histamine and mast cells in nerves Med exp (Basel) 4 180—182 1961
- UNANUE B Release processes in mast cells and their activation by injury Trans N Y Acad Sci 116 880—890 1964
- WAGGENER J P BURN M & BEGGS J The diffusion of ferritin within the peripheral nerve sheath An electron microscopy study J Neuropath exp Neurol 24 430—443 1965
- WARSHMAN B H Experimental study of diphtheric polyneuritis in the rabbit and guinea pig III The blood nerve barrier in the rabbit J Neuropath exp Neurol 20 35—77 1961
- WILHELM D L The mediation of increased vascular permeability in inflammation Pharmacol Rev 14 251—280 1962
- Chemical mediators I In The inflammatory process pp 389—425 Ed by B W Zweifach L Grant and R T McCluskey New York and London Academic Press 1965









ACTA PHYSIOLOGICA SCANDINAVICA  
VOL. 69 SUPPLEMENTUM 285



# STUDIES ON INTESTINAL DIPEPTIDASES

BY  
TOR LINDBERG

LUND 1



ORSTADIUS BOKTRYCKERI AKTIEBOLAG  
GÖTEBORG 1966

ACTA PHYSIOLOGICA SCANDINAVICA

VOL 69 SUPPLEMENTUM 285

From the Department of Physiological Chemistry

University of Lund Lund Sweden

STUDIES ON  
INTESTINAL DIPEPTIDASES

BY

TOR LINDBERG

LUND 1966

*Mr W F Salisbury revised the English text*

# CONTENTS

<i>Introduction</i>	5
<i>Determination of dipeptidase activity</i>	7
Earlier methods	7
Present method	7
Principle	7
Procedure	9
General considerations on the assay procedure	10
Unit of dipeptidase activity	10
<i>Previous studies on intestinal dipeptidases</i>	12
<i>Present investigation on intestinal dipeptidases</i>	15
General characteristics of intestinal dipeptidases	15
Effect of pH	16
Influence of bivalent metal ions	17
Distribution along the gastrointestinal tract and specific activity of intestinal dipeptidases	19
Distribution of dipeptidase activity along the gastrointestinal tract	19
Specific activities of intestinal dipeptidases in the various species	22
Prenatal development of intestinal dipeptidases	23
Dipeptidase activity in human foetus	24
Dipeptidase activity in the prenatal and postnatal life of rat	25
<i>General discussion</i>	27
Specificity of intestinal dipeptidases	27
Relation to other intestinal hydrolases	28
Concluding remarks	29
<i>Summary</i>	31
<i>References</i>	33

*This dissertation is based on the following publications*

- I Intestinal dipeptidases I Spectrophotometric determination and characterization of dipeptidase activity in pig intestinal mucosa (In collaboration with L Josefsson) *Biochim biophys Acta* (Amst) 1965 105 149-161
- II Intestinal dipeptidases II Distribution of dipeptidase activity in the small intestine of the pig (In collaboration with L Josefsson) *Biochim biophys Acta* (Amst) 1965 105 162-166
- III Intestinal dipeptidases III Characterization and determination of dipeptidase activity in adult rat intestinal mucosa (In collaboration with L Josefsson) *Acta physiol scand* 1966 66 410-418
- IV Intestinal dipeptidases Characterization, development and distribution of intestinal dipeptidases of the human foetus *Clin Sci* 1966 30 505-515
- V Intestinal dipeptidases Dipeptidase activity in the mucosa of the gastrointestinal tract of the adult human *Acta physiol scand* 1966 66 437-443
- VI Intestinal dipeptidases Development of dipeptidase activity in the small intestine of the rat as related to the development of the intestinal mucosa (In collaboration with Ch Owman) *Acta physiol scand* 1966 In press

The papers are referred to in the text by their respective Roman numerals

## INTRODUCTION

In the gastrointestinal tract, the various proteolytic enzymes hydrolyze the dietary protein as well as the endogenous protein (enzymes, mucus disrupted mucosal cells), which constitutes the major part of the protein in the intestinal lumen (for ref see Nasset 1964). Results from several investigations since those of van Slyke and Meyer 1912 and 1913 have given rise to the opinion that protein is completely hydrolyzed to its constituent amino acids before leaving the small intestine. Fisher in 1954 pointed out that no direct evidence supporting this theory had so far been presented. However, later and more conclusive investigations (Levenson, Rosen and Upjohn 1959; Dawson and Holdsworth 1962; Dawson and Porter 1962) have corroborated the earlier opinion, and it is now widely accepted that by far the largest part of the protein enters the blood from the gut as free amino acids.

Knowledge about the action of such enzymes as pepsin, trypsin, and chymotrypsin that take part in the initial hydrolysis of the protein is now well established (cf Bovey and Yanari 1960; Desnuelle 1960 a, b; Hill 1965). The further hydrolysis of the protein is continued by the action of the well characterized carboxypeptidases from pancreas and by leucine aminopeptidase from intestinal mucosa (cf Neurath 1960; Smith and Hill 1960). However, there are in the intestinal mucosa, other enzymes associated with the final digestion of the protein. These enzymes can be divided into two groups: polypeptide splitting enzymes (Smith 1951) and dipeptide splitting enzymes.

Several investigations during the past 60 years have demonstrated the dipeptide splitting activity of mucosal extract (see p. 12-13). It has also been shown that this enzyme group acts only on dipeptides: a free carboxyl group and a free amino group adjacent to the peptide bond are necessary for its action (for ref see Smith 1951). These enzymes can therefore be classified as dipeptidases (dipeptide hydrolases EC 3.4.3). As far as is known, tripeptide splitting enzymes have no or only very weak activity for dipeptides (Smith 1951, 1960). Concerning leucine aminopeptidase it has been demonstrated that purified preparations of this enzyme can

split some dipeptides, although at a much lower rate than their corresponding amide compounds (Smith and Hill 1960). The important role of the intestinal dipeptidases in the final digestion of the protein is apparent from the investigations of Agar, Hird and Sidhu (1953), Newey and Smyth (1959, 1960), and Wiggins and Johnston (1959), who showed that, *in vitro*, dipeptides (except for a small amount of glycylglycine) are hydrolyzed to their constituent amino acids before or during their transfer through the intestinal wall.

Lack of methods suitable for assaying the activity in crude tissue extracts and the instability of the enzymes have hampered the studies on the intestinal dipeptidases. In relation to other known proteolytic enzymes in the gut, relatively little information is available in the literature about the biochemical and physiological properties of this group of enzymes. Moreover, there is no information about the occurrence and distribution of the intestinal dipeptidases in man; thus, we know nothing about their significance in various pathological conditions of the gastrointestinal tract. Results from recent studies on gluten induced enteropathy (Frazer 1962, Messer, Anderson and Hubbard 1964, Bronstein, Haeflner and Kowlessar 1966) suggest that this disease may be caused by a congenital defect of some peptidase(s) in the intestinal mucosa. Before this can be definitely proved, however, it is necessary to get more information about the peptidases in the intestinal mucosa under physiological conditions.

For this purpose, a study of dipeptidase activity in extracts of intestinal mucosa, from various aspects, was started in our laboratory, as a rapid and simple spectrophotometric assay method had been devised (Josefsson 1964, 1). This method utilizes the strong absorption of the peptide bond in the low ultraviolet range. By the use of ethanol with negligible absorption down to 210 m $\mu$  as precipitating agent, the method allowed determination of dipeptidase activity in crude tissue extracts.

Our knowledge about the intestinal dipeptidases has been derived from investigations either of some single problems of the properties of the enzymes or of a single animal species. The literature lacks collected information obtained by the same assay method concerning the intestinal dipeptidases. The present study was therefore directed to a general survey of the main properties, of the distribution along the gut, and of the development of some dipeptidase activities in the intestinal mucosa. Knowledge about the main properties of this enzyme group is important for further studies in this sphere. By extending the study to three different species, we have obtained a comparative analysis of the biochemical and physiological properties of the enzymes.

# DETERMINATION OF DIPEPTIDASE ACTIVITY

## *Earlier methods*

The methods previously used in studies of dipeptidase activity are principally based on the determination of either the free carboxyl groups or the free amino groups formed when dipeptides are hydrolyzed. Macro methods were in use during the first decades of this century e.g. the formoltitration method of Sørensen (1908) and the amino nitrogen determination of van Slyke (1912). These were unsuitable for large series of enzyme analysis. A great improvement followed the introduction of micro methods such as that of Grassmann and Heyde (1929) (measurement of liberated carboxyl groups by titration with alcoholic KOH) and that of Linderstrøm Lang and Holter (1931) (titration of amino nitrogen with alcoholic HCl in acetone solution Linderstrøm Lang 1927). These two methods, as well as the ninhydrin method described by Moore and Stein (1948, 1954) which, based on the colour reaction between ninhydrin and  $\text{NH}_2$  groups, determines the amino acids photometrically, are still extensively used in assays of dipeptidase activity in crude tissue extracts and partially purified preparations. However, because these methods are non specific, they are adequate only when highly purified enzyme preparations are used. Further, although they have been classified as micro methods they require a relatively large amount of substrates. In 1938, Linderstrøm Lang and Lanz described a dilatometric micro method for peptidase activity, whose accuracy was about 50 times that of the earlier reported micro titrimetric method (Linderstrøm Lang and Holter 1931). This method, however, proved too laborious to be used in routine analysis.

Various modifications of the mentioned methods have also recently been used for assaying dipeptidase activity (see for example, Connell Dixon and Hanes 1955, Rademaker and Soons 1957, Glasser and Hanson 1962, Bryce and Rabin 1964, Matheson and Tattrie 1964).

## *Present method*

*Principle* Dipeptides have been known to exhibit a strong absorption in the low ultraviolet range in contrast to most of the amino acids (Magill



Steiger and Allen 1937 Saidel, Goldfarb and Waldman 1952, Saidel 1955) This strong absorption is mainly due to the peptide bond, a condition that has been utilized for determining protein concentration (for ref see Wetlaufer 1962) The difference in absorption between dipeptides and amino acids offers a possibility, already pointed out by Saidel in 1955, for studying the dipeptide splitting activity, because the absorption will decrease as peptide bonds are split This condition was utilized by Binkley and Torres (1960) in assaying leucine aminopeptidase activity in partially purified preparations, and by Schmitt and Siebert (1961, 1964) who studied some dipeptidase activities in extracts from pig and rat kidney and from cod muscle However, as crude tissue extracts and partially purified preparations contain a relatively large amount of substances—for example proteins and carboxylic acids, which have a high absorption in the low ultraviolet range and thus interfere with the determination—this method has *a priori* the same limitations as the earlier ones mentioned above

For a spectrophotometric assay method to be useful, therefore these disturbing substances had to be eliminated Ethanol with negligible absorption down to 210 m $\mu$  proved suitable for this purpose it did not influence the differences in the absorption between the dipeptides and amino acids (I, III) This was demonstrated by comparing the spectra of the dipeptides and of their constituent amino acids—obtained in aqueous solution—with the corresponding spectra obtained from the digest mixture made according to the standard assay procedure (see below) and precipitated with ethanol at zero time and read against water It was also found that the absorbancy of the amino acids did not change during the incubation with active enzyme solution or with water For this reason any occurring digestion of the protein or polypeptides present in the enzyme solution did not influence the absorbancy Moreover, the absorbancy of the dipeptides was unchanged after incubation with inactivated enzyme solution or with water Therefore the decrease of the absorbancy obtained when dipeptides were incubated with enzyme preparation must be ascribed to the splitting of the peptide bonds in the dipeptides

When work is done in the low ultraviolet range, some instrumental limitations exist (Saidel Goldfarb and Kalt 1951 Tombs Souter and MacLagan 1959) The concentrations of the various dipeptides were thus chosen to give not too high absorbancies as otherwise the readings would be difficult and uncertain At these concentrations, suitable difference absorbancy of about 0.8 was found at the wavelength of 220 m $\mu$  At this wavelength the absorbancy of the blanks containing the amino acids was

low, allowing a narrow slit width of the spectrophotometer. These procedures reduced the risk of interference from stray light occurring in the low ultraviolet range (for ref. see above)

**Procedure:** The standard assay procedure, described in detail in ref. 1 is briefly outlined here

Aqueous solutions of the various dipeptides in appropriate concentrations served as substrates, aqueous solutions of the analogous amino acids in equimolar concentrations served as blanks

The enzyme solution was prepared at 4°. Mucosa was scraped off the small intestine and homogenized in 0.1 M NaCl. After standing for 30 minutes the homogenate was centrifuged at 4° for 30 minutes at 27 000  $\times g$ . The supernatant was used as enzyme solution after suitable dilution with water.

20  $\mu$ l of the enzyme solution was added to an equilibrated (40°) mixture containing 50  $\mu$ l of dipeptide solution and 100  $\mu$ l of buffer solution (0.15 M borate or phosphate buffer) and thoroughly mixed. The incubation was performed at 40° and after suitable time 1.3 ml of ethanol-water (99:1 v/v) was added and mixed with the digest mixture. This procedure precipitated high molecular substances and interrupted the hydrolysis. The obtained precipitate was centrifuged down (5000 r.p.m. for 20 minutes) and the absorbancy of the supernatant was measured in a semi-microcuvette at 220 m $\mu$  with a spectrophotometer.

Samples containing the analogous amino acids, in equimolar concentration instead of dipeptides but treated identically in every other respect, were used as blanks.

In a large number of estimates with the various enzyme concentrations used, it was invariably found that the absorbancy of the digest mixture containing amino acids did not change during incubation. Because of this, in the later part of the study the time blanks were mostly eliminated, being included only at irregular intervals as controls and when a new source of enzyme preparation was used.

Under the conditions used, we found a zero order reaction rate of the initial hydrolysis of the five dipeptides studied with extracts from pig rat, and human intestinal mucosa. The hydrolysis of glycylglycine and glycyl-L-leucine followed a linear course until 30% and 40% respectively of the substrate were split whereas L-alanyl-L-glutamic acid, L-alanyl-L-proline, and glycyl-L-valine were hydrolyzed in a linear course until within 60-70% of completion.

By adding various concentrations of the enzyme solution, a linear cor

relation was found to exist between the rate of the hydrolysis of the various dipeptides and the enzyme concentrations used (within a 4 fold range)

*General considerations on the assay procedure* The present method is rapid and simple, it requires small amounts of substrate. It has also a high sensitivity, allowing the hydrolysis of  $5 \times 10^{-4} - 10^{-3}$  mmole of dipeptide to be measured, and a slight modification of the procedure allows further increase of the sensitivity, as recently shown in our laboratory (Josefsson, Norén and Sjöström—in preparation). The incubation time can be varied up to about 80 minutes without influencing the linear course of the hydrolysis. At longer incubation times, the linearity was lost probably because of inactivation of the enzyme. Variations of the enzyme concentration did not influence the absorbancy at zero time. Moreover, a variation of the amount of water in the incubation mixture within a range of  $\pm 20\%$  had no effect on the absorbancy. The slight influence of the buffer ions on the absorbancy is demonstrated by the finding that a variation of the concentration of the buffer salts within a range of  $\pm 70\%$  did not change the absorbancy at zero time more than  $\pm 10\%$ . Increasing or decreasing the ethanol volume by about 20% changed the absorbancy less than 10%.

The accuracy of the method was found to be as high as  $\pm 1\%$ —in experienced hands.

These advantages of the method together with the fact that it permits assaying dipeptidase activity in crude tissue extracts not only from the intestinal mucosa but also from other sources (Josefsson, Noren and Sjöström—in preparation), make the procedure very suitable for routine analysis.

However, the method is limited in that hydrolysis of dipeptides containing aromatic amino acids cannot be measured, because these amino acids exhibit a high absorbancy in the low ultraviolet range (for ref. see Wetlaufer 1962).

Routine control of the absorption of the various reagents used is necessary. The addition of substances with a high absorption in the low ultraviolet range may disturb the readings.

### *Unit of dipeptidase activity*

According to the recommendations of the Commission on Enzymes of the International Union of Biochemistry (1961) 1 unit is defined as the activity hydrolyzing 1  $\mu$ mole of dipeptide per minute at 40°.

When working with biological material, it is difficult to find a satisfactory reference substance to relate the enzyme activity to. Theoretically, the use of the number of homogenated epithelial mucosal cells as a reference must be the one most free from objection. However, that is impossible in this type of study. It was decided therefore to relate the enzyme activity to the total amount of nitrogen in the added enzyme solution, determined by the micro Kjeldahl procedure. The specific activity of the dipeptidases has thus been expressed as units per mg N present in the enzyme solution.

## PREVIOUS STUDIES ON INTESTINAL DIPEPTIDASES

Cohnheim demonstrated in 1901 that extract of intestinal mucosa decomposes pepton. This proteolytic component of mucosal extract was called erepsin.

A few years later, Abderhalden and Teruuchi (1906) and Euler (1907 a, b) reported that glycylglycine was split by the action of bovine and of pig intestinal mucosa. The effect of pH on this reaction was shown by Dernby (1917), who found a pH optimum of about 7.4 for the glycylglycine splitting activity of pig erepsin. This finding was later confirmed by von Euler and Josephson (1926 a). In another report, von Euler and Josephson (1926 b) demonstrated that the glycylglycine splitting activity of pig erepsin was inhibited if the amino group of the dipeptide was benzoylated. The authors concluded that a free amino group in the dipeptide was essential for the enzyme action. The problem of the specificity of the dipeptidase action was further studied by Bergmann and co-workers. Bergmann *et al.* (1935) confirmed that the presence of a free amino group was necessary; they showed that the enzyme required also a free carboxyl group for its action. This condition had earlier been found valid for yeast dipeptidase (Grassmann and Dyckerhoff 1928).

During the first decades after Cohnheim's discovery, the action of erepsin was generally believed to be due to a single enzyme. The first attempts to fractionate erepsin were made by Waldschmidt Leitz, Balls and Waldschmidt Graser (1929) who reported that their pig intestinal extract consisted of a polypeptidase and a dipeptidase. As substrates DL leucylglycylglycine and DL leucylglycine were used. In 1929, Linderström Lang and Linderström Lang and Sato demonstrated two different activities in pig intestinal mucosa: a leucylpeptidase (substrate: leucylglycine) and a dipeptidase (substrate: glycylglycine) indicated by different pH optima and stability. These activities were reported to be distinct from the mentioned polypeptidase. The leucylpeptidase was later called leucine aminopeptidase (Smith and Bergmann 1944). In 1936, Johnson, Johnson and Peterson made the important observation that leucylpeptidase was activated by  $Mg^{2+}$  ions. The effect of bivalent metal ions was further

studied by Berger and Johnson (1939), who reported that the hydrolysis of leucylglycine was activated by  $Mn^{+}$  ions to an even higher degree. They also observed that  $Mn^{+}$  ions and, above all,  $Co^{2+}$  ions markedly accelerated the hydrolysis of glycylglycine. The influence of various bivalent metal ions on dipeptidase activities in among other sources, extracts from rabbit and guinea pig intestine was further studied by Maschmann (1941 a, b). From the results of the studies on activation and specificity of various dipeptide splitting activities (Gailey and Johnson 1941), Johnson and Berger (1942) suggested that erepsin contained a number of dipeptidases. Observations supporting this suggestion had earlier been reported by Grassmann von Schoenebeck and Auerbach (1932) who found that L-prolylglycine was split by an enzyme prolinase in erepsin suggested as being different from other known enzymes and as acting differently. Bergmann and Fruton (1937) observed that glycyl L-proline was hydrolyzed by erepsin. This enzyme activity, called prolidase, was also suggested as being distinct from other enzyme activities in erepsin. Prolidase was also studied by Smith and Bergmann (1944) who found that the enzyme was influenced by bivalent metal ions, as were the other dipeptidases.

Detailed studies of the glycylglycine and glycyl L-leucine splitting activities in extracts from various tissues such as pig intestinal mucosa, human uterus and rat muscle, were carried out by Smith who in 1951 summarized the results in a review on the specificity of certain peptidases. On the basis of these investigations he suggested (1948 a) that the glycylglycine dipeptidase activity was extremely specific and later (1948 b) showed that a dipeptidase hydrolyzed glycyl L-leucine since substituting the amino group or the carboxyl group in the dipeptide prevented enzyme action. Various bivalent metal ions influenced specifically the glycyl L-leucine dipeptidase.

Intestinal dipeptidases have in recent years been studied by Robinson and Shaw (1960). Using rat intestinal mucosa they investigated the effect of pH and bivalent metal ions on the activities on L-leucylglycine, glycylglycine, glycyl L-leucine, and glycyl L-alanine. The distribution of the activities along the gut was also studied and the ileum was found to contain the highest activities. Robinson (1960) demonstrated that glycylglycine and glycyl L-valine dipeptidase activities were low in the large intestine. He studied the distribution of L-leucylglycine and glycylglycine dipeptidase activities in subcellular fractions of the rat intestinal mucosa (1963) and found that 80-90 % of the activities were left in the supernatant fraction.

To summarize The previous investigations have demonstrated that the intestinal mucosa contains an unknown number of enzymes acting on dipeptides, that the enzymes hydrolyze the peptide bond of the dipeptide only if free terminal amino groups and carboxyl groups are present—classifying them as dipeptidases, and that bivalent metal ions influence the action of the enzymes Moreover, the general opinion is that the intestinal dipeptidases have a high specificity, although the enzymes have not yet been purified

Dipeptidase activities are found not only in the mucosa of the gastrointestinal tract, but have been demonstrated in all animal tissues hitherto examined, for example, liver, and kidney (e.g. Maschmann 1941 a, b, Abderhalden 1943 Rademaker and Soons 1957, Hanson and Blech 1959, Wilcox and Fried 1963, Vescia and Traniello 1964, Patterson *et al* 1965), uterus and muscle (Smith 1948 a, b) placenta (Vescia and Fidanza 1948, Zoch 1965), and the lense of the eye (Glasser and Hanson 1963) Erythrocytes, leukocytes lymphocytes, and serum contain dipeptidase activities (e.g. Adams, McFadden and Smith 1952, Fleisher 1953, 1955, Haschen 1961 1963), which have also been found in micro organisms and plants (for ref. see Johnson and Berger 1942) In fact most of the investigations on dipeptidases reported so far have been performed, especially in recent years, on extracts from tissues other than the intestine Although Smith (1951) observed a difference concerning the stability and the influence of bivalent metal ions of glycylglycine and glycyl L leucine dipeptidase from various tissues, it has not yet been proved that the activity from various tissues against the same dipeptide is related to different enzyme molecules

# PRESENT INVESTIGATION ON INTESTINAL DIPEPTIDASES

The investigation was made on pig, rat, and man. The pig, which nutritionally resembles man, was chosen because large amounts of mucosa can easily be obtained. Thus, the intestinal mucosa of this animal is suitable for purification studies that require large amounts of enzyme material. The rat is a convenient laboratory animal and has been extensively used in intestinal absorption studies. Clinically, knowledge of the various properties of the intestinal dipeptidases in man is of great importance, especially as it has now been possible through the development of biopsy technique to study the function of the intestinal mucosa in various pathological conditions.

When selecting substrates from the large number of naturally occurring dipeptides, we considered three different aspects: the dipeptides would theoretically be split specifically; they should be easily obtainable commercially; and finally the activities on some of the dipeptides should have been investigated earlier. On these grounds, we chose the following five dipeptides: L-alanyl-L-glutamic acid, containing the acidic amino acid glutamic acid; L-alanyl-L-proline, an imidodipeptide; glycylglycine, the most simple dipeptide, whose hydrolysis has been studied before and is suggested to be very specific (see p. 13); glycyl-L-leucine, whose hydrolysis has also been studied before (see p. 13); and glycyl-L-valine, whose structure differs little from glycyl-L-leucine. This dipeptide was included in order to study whether any resemblance existed between the activities on these two substrates.

The present study can be divided into three parts:

- 1) general characteristics of intestinal dipeptidases
- 2) distribution along the gut and specific activity of intestinal dipeptidases
- 3) development in the prenatal life of intestinal dipeptidases

The results are given in the following paragraphs:

## *General characteristics of intestinal dipeptidases*

The results reported below are based on experiments performed on at least three different intestines from each species.



Table I Comparison of pH optima for dipeptidase activities in pig rat and human intestinal mucosa

Substrate	Pig	Rat		Man	
		Foetus	Adult	Foetus	Adult
L-Alanyl L-glutamic acid	7.4	7.2	7.3	7.5	7.3
L-Alanyl L-proline		7.0	7.0	7.0	7.0
Glycylglycine	7.9	7.5 <sup>1</sup>	7.5 <sup>1</sup>	7.6	7.8
Glycyl L-leucine	7.9	7.4	7.5	7.9	7.6
Glycyl L-valine	7.6	7.5	7.4	7.8	7.6

In the presence of  $\text{Co}^{+}$  ions

**Effect of pH** Various phosphate and borate buffer solutions were used for studying the influence of pH on the dipeptidase activities. The pH values in the digest mixture (measured by glass electrode) before and after digestion were found to differ less than 0.1, which agrees with the observation of Smith (1948a) when studying glycylglycine dipeptidase. When determining the pH optima, we chose the initial pH values in the digest mixtures. Table I records a comparison of the obtained pH optima for the various activities in the different species including rat and human foetus (I-III, IV-V, VI). As seen from the table, the pH optima for the various dipeptide splitting activities varied between 7.0 and 7.9. Some smaller differences of the pH optima between the various species are also evident.

In the literature reports are available of the influence of pH on the activities on glycylglycine, glycyl L-leucine and glycyl L-valine. Dernby (1917) found in extracts from pig intestinal mucosa the pH optimum for glycylglycine dipeptidase activity to be 7.8. von Euler and Josephson (1926a) found it to be 8.0, whereas Linderström-Lang and Sato (1929) reported it to be 7.3. Robinson and Shaw (1960) observed in rat intestinal mucosa two pH optima, 7.4 and 8.2, which finding together with an observed difference of the effect of  $\text{Mn}^{+}$  ions, was interpreted as indicating that two enzymes were present.

Studying rat liver glycylglycine dipeptidase, Wilcox and Fried (1963) also found among other variables studied two pH optima, 8.1-8.2 in the residual system and 8.5 in the  $\text{Co}^{+}$  ion or  $\text{Mn}^{+}$  ion system. From other tissues, such as human uterus (Smith 1948a, pH optimum 7.6), human erythrocytes (Haschen 1963, pH optimum 8.0) and human leucocytes

(Fleisher 1955 pH optimum 7.7), only one pH optimum was observed, whereas Fleisher (1953) studying normal serum found three optima in the  $\text{Co}^{2+}$  ion system (6.2-6.3, 6.8, and 8.0-8.3) but only one optimum in the  $\text{Mn}^{+}$  ion system (7.4). In the present study, only one pH optimum for glycylglycine dipeptidase activity in the three species investigated was observed in the  $\text{Co}^{+}$  ion system or in the residual system (i.e. no metal ions added to the crude tissue extract).

Investigating glycyl L leucine dipeptidase activity in rat intestinal mucosa Robinson and Shaw (1960) found two pH optima (7.6 and 8.6). This could not be confirmed in the present study, but in rat, the activity declined slowly in the pH range between 7.5 and 8.5.

In extracts from other tissues, such as bovine lense of the eye (Glässer and Hanson 1963 pH optimum 7.5), human erythrocytes (Haschen 1961 pH optimum 8.2) and normal serum (Fleisher 1953 pH optimum 9.4), only one pH optimum was observed.

Robinson (1960) reported the pH optimum of glycyl L valine dipeptidase activity in rat intestinal mucosa to be 8.4; this value differs distinctly from that observed in the present study.

It has been observed that the rate of the hydrolysis of glycyl L leucine is accelerated when phosphate buffer is used (Smith 1948 b, Haschen 1961, Glässer and Hanson 1963), probably owing to the binding of the inhibitory  $\text{Ca}^{+}$  ions to phosphate (Smith 1948 b). At a comparison of the rate of the hydrolysis of the various dipeptides in the phosphate buffer system with that of the borate buffer system at the same final pH, no differences were found in the present study.

**Influence of bivalent metal ions** As the dipeptidases are influenced by bivalent metal ions (Smith 1951) the effect of  $\text{Co}^{+}$ ,  $\text{Mn}^{+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Zn}^{+}$  ions on the hydrolysis was studied in the various species (I, III, IV, V and VI). From Table II, which gives a summary of the results, it is apparent that the pattern of the effect of the four metal ions on the hydrolysis differed from one dipeptide to another. However, the difference between glycyl L leucine dipeptidase activity and glycyl L valine dipeptidase activity was not so marked. At a comparison of the influence of the bivalent metal ions on the activities in the various species, it should be observed that for glycylglycine, glycyl L leucine and glycyl L valine dipeptidase activities different buffer systems were used in some of the species. It has been shown that the anions in the buffer system affect the influence of mainly the  $\text{Zn}^{2+}$  ions (Smith 1948 b, I, III). In the phosphate buffer system, the inhibitory effect of the  $\text{Zn}^{+}$  ions was less than in the

Table I Comparison of pH optima for dipeptidase activities in pig rat and human intestinal mucosa

Substrate	Pig	Rat		Man	
		Foetus	Adult	Foetus	Adult
L-Alanyl L-glutamic acid	74	72	73	75	73
L-Alanyl L-proline		70	70	70	70
Glycylglycine	79	75 <sup>1</sup>	75	76 <sup>1</sup>	78
Glycyl L-leucine	79	74	75	79	76
Glycyl L-valine	76	75	74	78	76

<sup>1</sup> In the presence of Co<sup>++</sup> ions

**Effect of pH** Various phosphate and borate buffer solutions were used for studying the influence of pH on the dipeptidase activities. The pH values in the digest mixture (measured by glass electrode) before and after digestion were found to differ less than 0.1, which agrees with the observation of Smith (1948a) when studying glycylglycine dipeptidase. When determining the pH optima, we chose the initial pH values in the digest mixtures. Table I records a comparison of the obtained pH optima for the various activities in the different species including rat and human foetus (I, III, IV, V, VI). As seen from the table, the pH optima for the various dipeptide splitting activities varied between 7.0 and 7.8. Some smaller differences of the pH optima between the various species are also evident.

In the literature reports are available of the influence of pH on the activities on glycylglycine, glycyl L-leucine and glycyl L-valine. Dernby (1917) found in extracts from pig intestinal mucosa the pH optimum for glycylglycine dipeptidase activity to be 7.8. von Euler and Josephson (1926a) found it to be 8.0, whereas Linderström-Lang and Sato (1929) reported it to be 7.3. Robinson and Shaw (1960) observed in rat intestinal mucosa two pH optima 7.4 and 8.2 which finding, together with an observed difference of the effect of Mn<sup>++</sup> ions was interpreted as indicating that two enzymes were present.

Studying rat liver glycylglycine dipeptidase Wilcox and Fried (1963) also found among other variables studied two pH optima 8.1-8.2 in the residual system and 8.5 in the Co<sup>++</sup> ion or Mn<sup>++</sup> ion system. From other tissues, such as human uterus (Smith 1948a pH optimum 7.6), human erythrocytes (Haschen 1963 pH optimum 8.0), and human leucocytes

1955 Togasawa and Katsumata 1956 Rademaker and Soons 1957, Robinson and Shaw 1960 Haschen 1963, Wilcox and Fried 1963)

Concerning the *glycyl L leucine* dipeptidase activity, the inhibitory effect of  $\text{Co}^{+}$  ions and  $\text{Mn}^{+}$  ions has previously been reported in the literature (Maschmann 1941 b Smith 1948 b, Robinson and Shaw 1960, Haschen 1961) However, Smith (1948 b) reported that after dialyzing and filtering enzyme solution obtained from pig intestinal mucosa the activity was specifically activated by  $\text{Mn}^{+}$  ions In crude extracts of intestinal mucosa, Smith (1948 b) and Robinson and Shaw (1960) observed that the  $\text{Mg}^{+}$  ions also inhibited the reaction this could not be confirmed in the present study The inhibitory effect of  $\text{Zn}^{+}$  ions on the hydrolysis of *glycyl L leucine* has previously been observed in studies on intestinal mucosa (Smith 1948 b, Robinson and Shaw 1960) as well as on other tissues (Fleisher 1953 Glasser and Hanson 1963) Nevertheless, this activity in human uterus and rat muscle is activated by these ions in the presence of phosphate buffer (Smith 1948 b)

Concerning the other dipeptidase activities studied no earlier data about the influence of bivalent metal ions have been found in the literature

When the significance of the obtained findings concerning the influence of the bivalent metal ions on the various activities is considered, it must be remembered that the experiments were performed on crude tissue extracts which from the beginning contain an unknown concentration of these ions If we add the various bivalent metal ions in varying concentrations their influence on the reaction might however, be satisfactorily studied in spite of our lack of knowledge about their true concentration in the digest mixture Little is known about the activity of the intestinal dipeptidases where metal ions are not present Josefsson and Sjostrom (1966) have shown that when the mucosa is homogenated in 0.005 M EDTA the activity of *glycyl L leucine* dipeptidase is about one third of that when the mucosa is homogenated in 0.1 M NaCl

#### *Distribution along the gastrointestinal tract and specific activity of intestinal dipeptidases*

*Distribution of dipeptidase activity along the gastrointestinal tract* Various regions of the gastrointestinal tract are known to differ in their capacity to absorb the various nutrients By labelling the dietary protein its absorption in man has been shown to occur in the upper part of the small intestine shortly after ingestion (Borgstrom *et al* 1957, Crane and Neuber

ger 1960) However, so far, there has been no direct study on the dipeptide splitting capacity of the various regions of the human gut The only studies of this problem are those of Robinson and Shaw (1960) and Robinson (1960), who investigated the distribution of some dipeptidase activities in the rat intestine

The dipeptide splitting capacity was therefore determined in various levels of pig, rat, and human gastrointestinal tract (II, III, IV, V, VI) In order to compare the activities in the various sections examined the specific activities of the dipeptidases were calculated (units of dipeptidase activity per mg total nitrogen present in the enzyme solution)

The results showed that in pig (II) and adult man (V) the activities against the five dipeptides studied were very low in the proximal part of the duodenum In its distal part, the activities rose rather sharply and reached in pig, a maximum level in the jejunum and in the proximal part of the ileum In adult man, the activities were even higher in the terminal part of the ileum, whereas in pig the activities declined in this region In rat (III), however, the activities were high, even in the proximal part of duodenum Maximum values were found in the jejunum and in the upper part of the ileum in three of the five intestines studied These findings agree with those of Robinson and Shaw (1960) However, no significant difference between the various regions of the rat intestine was found in the present study In fact, in two of the intestines the activities were as high, or even higher in the duodenum as in the more distal parts of the gut

The amount of dipeptidase activity in various parts of the developing rat small intestine was determined in connection with the study of the development of rat intestinal dipeptidases (VI) Owing to the small size of the gut in this early age proportionally larger sections had to be taken for the preparation of enzyme solution From the 19 day stage *post coitum* up to and including birth the activity on L-alanyl-L-proline was the same in the proximal third as in the middle and distal thirds whereas the activities on the other four dipeptides were higher in the distal two thirds of the small intestine In the postnatal stages up to the 3rd week *post partum* all the activities were slightly stronger in the middle or distal thirds of the small intestine

As fresh material is needed for the enzyme studies and as the investigation on adult man was performed on operation specimens it was impossible to determine the distribution of the activities along the whole length of the intestine in the same individual However, this was possible in the studies of the development of the enzyme activities in the human prenatal

life (IV) In human foetuses between 11 and 23 weeks of fertilization age, maximum dipeptidase activities were found in the proximal part of the gut, not in the middle and distal parts. Although this difference was not significant, it seems that the distribution of the activities change during the later period of development. It should be emphasized that proportionately larger sections of the human foetus intestine were investigated. However, the same high activities were found in the proximal part of the gut even when examining sections as small as four per cent of the total length of the small intestine.

A comparison of the distribution of the various dipeptidase activities revealed no significant difference. In pig and rat, the level of maximum glycylglycine dipeptidase activity tended to be proximally localized compared with the level of the other activities.

When we relate the findings concerning the distribution of dipeptidase activities in the human small intestine to our knowledge about the localization of the digestion and absorption of dietary protein over the intestinal length in man (Borgstrom *et al.* 1957, Crane and Neuberger 1960) it is apparent that the activities in the distal duodenum and the upper jejunum are adequate for accomplishing the final digestion of the given protein. The physiological role of the large amount of dipeptidase activity found in the distal jejunum and in the ileum is probably that of accomplishing the final digestion of the endogenous protein which as known (for ref. see Nasset 1964) forms by far the largest part of the protein in the lumen and which is hydrolyzed and absorbed in the distal parts of the small intestine.

The dipeptidase activities were determined also in the gastric and the large intestinal mucosa in rat (III), human foetus (IV), and adult man (V). The activities in the stomach were generally low compared with those found in the jejunum/ileum. Equally low activities were found in the large intestinal mucosa of rat and adult man. The large intestine of human foetuses however contained the same amount of dipeptidase activities as the foetal small intestine. The obtained difference between the specific activities in the adult and the foetal large intestine was significant for the activities on L-alanyl-L-proline ( $p < 0.005$ ), glycyl-L-leucine ( $p < 0.05$ ), and glycyl-L-valine ( $p < 0.05$ ) but not significant for L-alanyl-L-glutamic acid dipeptidase activity ( $p < 0.4$ ) as calculated according to Student's *t* test. (The values for glycylglycine dipeptidase activity could not be compared since, in human foetus, this activity was measured only in the presence of  $\text{Co}^{+}$  ions.) It should be pointed out that in adults only the mucosa was used as a source of the enzyme whereas in foetuses the

Table III Comparison of specific activity (units per mg nitrogen, mean values  $\pm$  SD) of intestinal dipeptidases from various species Enzyme solution obtained from jejunum  
Optimum pH

Substrate	Pig	Rat	Adult man <sup>1</sup>
L-Alanyl L-glutamic acid	35.4 $\pm$ 7.7 (n=6)	10.8 $\pm$ 3.8 (n=10)	26.3 $\pm$ 5.4 (n=3)
L-Alanyl L-proline		7.95 $\pm$ 0.83 (n=5)	6.09 $\pm$ 3.34 (n=2)
Glycylglycine*	6.61 $\pm$ 2.94 (n=11)	1.30 $\pm$ 0.60 (n=7)	8.86 $\pm$ 1.00 (n=3)
Glycyl L-leucine	129.2 $\pm$ 24.8 (n=7)	26.1 $\pm$ 6.4 (n=11)	232.3 $\pm$ 57.7 (n=3)
Glycyl L-valine	81.4 $\pm$ 32.5 (n=8)	21.5 $\pm$ 7.1 (n=10)	111.4 $\pm$ 13.6 (n=3)

<sup>1</sup> Values obtained from the three patients EA, EA and EA (V)

\* No metal ions added

entire intestinal wall was used. Thus, the obtained difference of the specific activities (units per mg nitrogen) are perhaps even greater. The cause of this decrease of the activities in the large intestinal mucosa from foetal to adult life is difficult to establish. An important difference between the adult and the foetal large intestine is the rich bacterial flora in the adult large intestine. It is perhaps possible that some proteolytic enzymes produced by micro organisms in the large intestine destroy the intestinal hydrolases and thereby reduce their activity. However, it should also be noted that villi-like projections are found in the large intestine in the foetal life period (Johnson 1913; Patzelt 1931, IV) and that these villi-like structures disappear during the later foetal period. To discover whether this disappearance is related to the decrease of the dipeptidase activities, it should be of interest to investigate intestines from newborn infants, but it has so far been impossible to get fresh material from this age.

*Specific activities of intestinal dipeptidases in the various species* To get an idea of the ability of the intestinal mucosa from the various species to split the dipeptides, the specific activities of the dipeptidases in jejunum were calculated. Table III presents a comparison of the values obtained (mean values  $\pm$  SD) for the various activities in the different species. If the various dipeptide splitting activities are first compared with one another, it is evident that the activities on glycylglycine and L-alanyl L-proline were low compared with the high activities on glycyl L-leucine and glycyl L-valine. The jejunum of pig and adult man contained about the same amount of activities with the exception of glycyl L-leucine dipeptidase activity which was highest in adult man. In rat, relatively low

Table IV Comparison of relative specific activity (related to glycylglycine dipeptidase activity) of intestinal dipeptidases from various species Enzyme solution obtained from jejunum Optimum pH

Substrate	Pig	Rat	Adult man <sup>a</sup>
L-Alanyl L-glutamic acid	5.36	8.31	2.97
L-Alanyl L-proline		6.12	0.69
Glycylglycine <sup>a</sup>	1.0	1.0	1.0
Glycyl L-leucine	19.5	20.1	26.2
Glycyl L-valine	12.3	16.5	12.6

Values obtained from the three patients EA EA and EA (V)

<sup>a</sup> No metal ions added

specific activities were found for four of the dipeptidases, whereas the fifth activity, L-alanyl L-proline dipeptidase was proportionately very high. This became more obvious when the relative specific activities were calculated (Table IV).

As little is known about the occurrence and concentrations of the various dipeptides in the dietary and the endogenous protein it is at present impossible to relate to the physiological need the obtained findings concerning the capacity of the mucosa to split the various dipeptides.

### *Prenatal development of intestinal dipeptidases*

It has long been established that the newborn infant has no difficulty in digesting and absorbing protein (*cf* Smuth 1959). The various proteolytic enzymes dealing with the digestion of the protein therefore appear to be present in sufficient amounts at birth. Previous investigations on the development of proteolytic enzymes in man have concerned pepsin (e.g. Keene and Hewer 1929, Werner 1948, Wagner 1958), trypsin (e.g. Ibrahim 1909, Keene and Hewer 1929, Werner 1948), and erepsin (Jaeggli 1907, Langstein and Soldin 1908, Tachibana 1927, Keene and Hewer 1929). The proteolytic activity of erepsin was estimated by the biuret method which does not permit a determination of the breakdown of dipeptides. In a short communication, Blum, Jarmoschkewitsch and Jakowtschuk (1936) reported that intestinal glycylglycine dipeptidase activity assayed by the micro titration method could be demonstrated as early as in the second month of development of the human foetus. However, so far, no detailed investigation into the prenatal development of the



Table V Comparison of specific activity (units/mg nitrogen mean values  $\pm$  SD) of intestinal dipeptidases in human foetus (11-23 weeks of fertilization age) and adult man. Enzyme solution obtained from jejunum Optimum pH

Substrate	Human foetus		Adult man <sup>a</sup>
	Entire intestinal wall	Mucosa (corrected mean values <sup>1</sup> )	
L-Alanyl L-glutamic acid	188 $\pm$ 98 (n=23)	26.3	26.3 $\pm$ 5.4 (n=3)
L-Alanyl L-proline	480 $\pm$ 212 (n=19)	5.28	6.09 $\pm$ 3.34 (n=2)
Glycylglycine <sup>a</sup>	552 $\pm$ 100 (n=4)	6.62	8.86 $\pm$ 1.95 (n=3)
Glycyl L-leucine	139.5 $\pm$ 57.0 (n=25)	167.4	232.3 $\pm$ 57.7 (n=3)
Glycyl L-alanine	81.4 $\pm$ 36.7 (n=21)	122.1	111.4 $\pm$ 13.6 (n=3)

For explanation see text

Values obtained from the three patients EA, EA and EA (V)

No metal ions added

intestinal dipeptidase activities in any species has been reported in the literature

*Dipeptidase activity in human foetus (IV)* The study included 35 foetuses obtained at legal abortions performed by hysterotomy. The fertilization age of the foetuses, determined by measuring the crown rump length (C-R length) varied from 11 to 23 weeks (corresponding to 6.2 cm to 21.5 cm C-R length Patten 1953). The dipeptidase activities were assayed in a section from the upper part of the small intestine (in foetuses smaller than 10 cm C-R length the proximal third or fourth of the gut; in larger foetuses in a 5 cm piece 10 cm from the pylorus). When the obtained specific activities of the various dipeptidases were plotted against the C-R length of the various foetuses, no significant difference was found between the values from the smallest compared with those from the largest. Thus in this period of foetal life no increase of the dipeptidase activities occurred.

The separate values of the various specific activities were therefore combined and the mean values  $\pm$  SD were calculated and compared (Table V) with those obtained from three adult jejunal mucosal samples. As the table shows the values of glycyl L-leucine and glycylglycine dipeptidase activities in adult mucosa were about 60 per cent higher whereas the other activities were only slightly higher. However as mentioned earlier because the foetal intestine was too thin to permit removal of the

mucosa in most of the foetuses, the entire intestinal wall was used for the enzyme preparation, whereas in the adult, only the mucosa was used. This difference might be partly responsible for the higher values in adults. Therefore, to test this, the dipeptidase activities in the mucosal samples and in samples from a consecutive section of the entire intestinal wall were assayed in some of the larger foetuses, where the mucosa could be scraped off, and the following relations between mucosal activities and whole wall activities were obtained for L-alanyl-L-glutamic acid 1.4, for L-alanyl-L-proline 1.1, for glycylglycine 1.2, for glycyl-L-leucine 1.2, and for glycyl-L-valine 1.5. With the aid of these figures, the specific activities in the foetal mucosa were calculated, the results are given in Table V. The activities on glycylglycine and glycyl-L-leucine remained somewhat lower in the foetuses but the difference is not significant. It can therefore be concluded that the dipeptidase activities are fully developed at the foetal age of 11 weeks.

Microscopical examinations of the structure of the mucosa in the actual foetal period revealed that it was well developed at this stage, which agrees with the findings of Johnson (1910) and Patzelt (1931).

*Dipeptidase activity in the prenatal and postnatal life of rat (VI)* In the study of the development of the dipeptidase activities in human foetuses, it was possible to get fresh material only from foetuses of 11 to 23 weeks fertilization age. In order to get an opportunity to study the development of the activities over a relatively longer period of foetal life, rat foetuses were investigated.

Three or four litters a day from and including day 15 *post coitum* up to birth (22–22½ days *post coitum*), were investigated: a total of 252 foetuses from 35 albino rats. The following postnatal stages were also included: newborn nonsuckled rats (6 litters), newborn suckled rats (about 6 hours of age, 3 litters) and finally rats from the third day and the second and third weeks of life (4 litters). Parallel with the enzyme analyses, microscopical examination of the mucosal structure was made in all the stages.

The investigation showed that in the period of mucosal cell proliferation (15 to 18 days *post coitum*) the dipeptidase activities were very low. Between the 18½ day stage *post coitum* and parturition, the activities increased uniformly and reached maximum values in the 22 day stage or in the newborn nonsuckled rats. This increase coincides with the differentiating period of the mucosa. But it is impossible to determine from this study what structural change in the mucosal cells is responsible for this

increase in the functional capacity of the intestine. From a morphological aspect, it is interesting to note that the brush border membrane appears in the 18 1/2 day stage *post coitum* and that the anlage of the crypts of Lieberkuhn is not observed until the activities become maximum.

The specific activities in newborn nonsuckled rats were about three times as high as those in adult rat. However, the activities on L-alanyl-L-glutamic acid, glycylglycine, glycyl-L-leucine and glycyl-L-valine fell significantly to the relatively low adult level only a few hours after birth, i.e. when the rats had sucked. The activity on L-alanyl-L-proline, on the other hand, declined successively in the postnatal period and first reached the adult level at the third week *post partum*. The cause of the abrupt decrease of the four activities is at present not clear. In the newborn period, several events occur: feeding, bacterial invasion, hormonal adjustment etc. The effect of rat colostrum on the dipeptidase activities was studied in some experiments. It was found that the activity on L-alanyl-L-proline was slightly inhibited, whereas the activities on the other dipeptides were considerably reduced. The nature of the inhibitory factor(s) in colostrum is unknown and requires further studies. The effect of bacterial invasion on the intestinal hydrolases has been studied by Dahlqvist, Bull and Gustafsson (1965). They found no difference between the disaccharidases in the small intestine of conventional adult rats and those in the intestine of germ free adult rats, whereas large differences of some activities were observed in the caecum and the large intestine. A study of the possible hormonal influence upon the enzyme activities has shown that corticosteroids can affect the development curves of the alkaline phosphatases (Moog 1962; Halliday 1959) and of the disaccharidases (Doell and Kretschmer 1964; Koldovsky and Chytil 1965).

At a comparison of the development of intestinal hydrolases in various species, it must be remembered that the stage of the structural development of the mucosa at birth differs in various species. Thus, in the present study, the structure of the mucosa in newborn rats was found to be comparable with that of human foetus of about 12 weeks fertilization age. In human foetuses, the differentiating period of the mucosa has been shown to occur between 18 mm and 55-60 mm C.R. length (Johnson 1910; Patzelt 1931), thus just prior to the foetal period actually studied. As the dipeptidase activities were well developed in the smallest foetuses investigated (62 mm C.R. length) and in view of the results from the study of developing rats, it seems reasonable to suggest that the development of the dipeptidase activities also in man coincides with the differentiating period of the mucosa.

## GENERAL DISCUSSION

*Specificity of intestinal dipeptidases* Although it is important to determine whether the various dipeptide splitting activities are related to different specific enzymes or to the action of a single enzyme, no extensive purification studies of the intestinal dipeptidases have so far been reported. Such studies have mostly been hampered by the lability of the enzyme activities. The different effects of pH and bivalent metal ions on the various dipeptidase activities observed in this investigation, as well as in others are not of course, direct proof that the activities are related to different enzyme molecules.

In a study on an approximately 2000 fold purified peptidase from swine kidney Vescia and Traniello (1964) found that this enzyme preparation, which was not homogeneous split L leucylglycine in the presence of  $Mn^{+2}$  ions, glycylglycine in the presence of  $Co^{+2}$  ions, and glycyl L-leucine in the absence of metal ions. However it was suggested that the different activities were related to a single enzyme, as no difference was found in their relative rates of hydrolysis during the purification procedure, in their pH optima and pH stability, and finally in their inactivation course. Patterson *et al* (1965), studying rat liver peptidases, could separate two enzymes with glycyl L-leucine splitting activity, glycylleucine aminohydrolase (S) and glycylleucine aminohydrolase (F), but they were unable to distinguish L-alanyl-glycine dipeptidase from glycylleucine aminohydrolase (F). However on the basis of the different shapes of the peaks of the enzyme activities after electrophoretic separation it was presumed that these two activities also were related to different enzymes. Recently Campbell *et al* (1966) purified a particulate dipeptidase from hog kidney. The purified fraction, which they found to be homogeneous in ultracentrifugation and in acrylamide gel electrophoresis experiments, catalysed the hydrolysis of several dipeptides but did not act on tripeptides and L-leucineamide.

In the studies on the intestinal dipeptidases we have repeatedly observed a different behaviour of especially L-alanyl-L-proline dipeptidase activity (e.g. influence of pH, metal ions and colostrum on the reaction, the disappearance of this activity in meconium from newborn infants (IV)

and the relatively high activity in intestinal contents (Josefsson, Lindberg and Öjesjö—in preparation)) which argues in favour of the suggestion that this dipeptide is split by a separate enzyme. It has recently been consistently demonstrated in purification studies on pig intestinal dipeptidases (Josefsson, to be published) that the activity on L-alanyl-L-proline can be completely separated from the activities on L-alanyl-L-glutamic acid, glycyl-L-leucine, and L-glutamyl-L-valine.

*Relation to other intestinal hydrolases* Concerning the distribution of the intestinal hydrolases along the gut, it is of interest to note that in man the distribution of the dipeptidase activities is similar to that of the disaccharidases. Thus, Auricchio *et al.* (1963) reported that in man the activities of the intestinal disaccharidases maltase, invertase, isomaltase and lactase were low in the proximal part of the duodenum whereas the jejunum and the ileum contained large amounts of the activities. The findings on pig in the present study resembled those of Dahlqvist (1961), who found in an adult pig low invertase, isomaltase, and maltase activities in the proximal duodenum, whereas they were high in the jejunum-ileum region. However, cellobias, lactase, and trehalase activities were strongest in the proximal part of the gut. In rat (Dahlqvist 1963), invertase, isomaltase, and trehalase activities were highest in the proximal two thirds of the small intestine whereas maltase activity was equally high throughout the entire length of the gut, and lactase activity highest in the middle third (Dahlqvist and Thomson 1964).

The above mentioned investigations on pig and rat suggest that the various disaccharidases differ in their distribution even in the same species in contrast to the condition found valid for the dipeptidase activities.

Regarding the development of the intestinal hydrolases, the disaccharidases appear early in human foetal life (Auricchio, Rubino and Murset 1965; Dahlqvist and Lindberg 1966) as do the dipeptidases. Dahlqvist and Lindberg (1966) studied the development of disaccharidase and alkaline phosphatase activities in the same human foetal material as used for the study of the development of the dipeptidase activities (IV). Invertase, isomaltase, and maltase were found to be well developed already at the foetal age of 11 weeks whereas trehalase developed from the 11th to the 23rd weeks and lactase even later in the foetal life. Although alkaline phosphatase activity increased from the 11th to the 23rd weeks of foetal life this activity was still below the adult level.

When the present findings on developing rats are related to earlier studies on the development of rat intestinal disaccharidases, the prenatal

developing curve for lactase activity (Alvarez and Sas 1961, Doell and Kretchmer 1962) is found to resemble that of the dipeptidase activities. In the postnatal period, the lactase activity successively decreases and reaches the low adult level in the fourth week *post partum* (Alvarez and Sas 1961, Rubino Zimbalatti and Auricchio 1964, Doell and Kretchmer 1962). The  $\alpha$  disaccharidases, however, are low or absent in the first two weeks *post partum* and thereafter increase rapidly to the adult level (Doell and Kretchmer 1963, 1964, Rubino *et al* 1964).

### *Concluding remarks*

The results from the present investigation have provided a basis for further studies on various problems concerning the intestinal dipeptidases, such as their clinical significance and the location of their physiological action.

By the peroral small intestinal biopsy technique a sufficient amount of mucosa is obtained for assaying dipeptidase activity and as the spectrophotometric assay method is suitable for routine analysis we are now able to make a more general study of the intestinal dipeptidases in relation to various disorders of the gut (Josefsson Lindberg and Norden 1966). Indirect evidence suggests that gluten induced enteropathy is perhaps due to a deficiency of one or more peptidases in the intestinal mucosa (Frazer 1962, Messer, Anderson and Hubbard 1964, Bronstein, Haeffner and Kowlessar 1966). Attempts by Messer, Anderson and Townley (1961) to demonstrate a deficient peptidase activity in duodenal biopsy specimens from coeliac children failed, however, although the specimens were reported as showing gross histological changes. It is of interest therefore to note that of the results hitherto achieved in our investigation (Josefsson Lindberg and Norden 1966) the dipeptidase activities in the mucosa taken from the region of flexura duodeno-jejunalis in patients with gluten induced enteropathy were significantly lower than those in a control material. At an extension of the investigation to children, the same results were obtained (Lindberg unpublished observations). Whether the low intestinal dipeptidase activities observed in the patients with gluten induced enteropathy are only a secondary phenomenon due to the atrophy of villi in these patients or whether they are a specific feature of this disease remains to be established.

The location of the physiological action of intestinal dipeptidases is still unsolved. In fact the various investigations directed on this problem have produced various results supporting the theory that the hydrolysis

occurs mainly in the lumen of the gut (Szafran, Szafran and Oleksy 1962), on the external surface of the mucosal cell membrane (Ugolev *et al* 1964 Ugolev 1965), just before entering the mucosal cell or very rapidly within the cell (Dawson and Holdsworth 1962), and finally, within the mucosal cell (Newey and Smyth 1960, 1962) We are now studying this problem in our laboratory Results hitherto obtained show that intestinal content collected from man and rat contains only small amounts of dipeptidase activities, except for L-alanyl L-proline dipeptidase activity (Josefsson, Lindberg and Öjesjö—in preparation) These results suggest that the hydrolysis of dipeptides occurring in the intestinal lumen is of minor importance in the final digestion of protein, with the possible exception of L-alanyl L-proline However it remains to be established whether the dipeptides are hydrolyzed outside the mucosal cell membrane or absorbed and split intracellularly

## SUMMARY

*A rapid and simple spectrophotometric assay method for assaying dipeptidase activity in crude extracts of intestinal mucosa is described. By this method, the activities on five dipeptides—L-alanyl-L-glutamic acid, L-alanyl-L-proline, glycylglycine, glycyl-L-leucine and glycyl-L-valine, in the mucosa of the gastrointestinal tract of pig, rat and man were determined.*

The influence of pH on the various activities was studied and the pH optima were found to vary from 7.0 to 7.9. The pH optimum for each dipeptide splitting activity differed slightly from one species to another.

The rate of the hydrolysis of the dipeptides, except L-alanyl-L-proline was generally influenced in all the species by the bivalent metal ions  $\text{Co}^{+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Zn}^{+}$ , whereas the  $\text{Mg}^{+}$  ions generally did not affect the dipeptide splitting activities. It was also found that the influence pattern of the bivalent metal ions on the hydrolysis differed from one dipeptide to another.

The distribution of the dipeptidase activities along the gastrointestinal tract of pig, rat foetus, adult rat, human foetus and adult man was investigated. Generally low activities were found in the stomach. The jejunum and the ileum of all the species contained large amounts of dipeptidase activities. In the proximal duodenum of pig and adult man, low activities were found in contrast to those found in adult rat and human foetus. The large intestine in adult rat and adult man contained small amounts of activities, whereas in that of the human foetus they were as high as in the small intestine and significantly higher than those in the adult large intestine.

With one exception the specific activities of the intestinal dipeptidases were of about the same magnitude in the pig as in the adult human jejunal mucosa, the glycyl-L-leucine dipeptidase activity was strongest in adult man. The rat jejunum however had relatively low specific activities except for L-alanyl-L-proline dipeptidase, which was even higher in rat than it was in adult man.

Studies on the development of the intestinal dipeptidases revealed that



in man the activities were fully developed at 11 weeks of fertilization age, 16 in the smallest foetus studied. The structure of the mucosa at this stage was well developed. The study of developing rat revealed that the dipeptidase activities increased in the differentiating period of the mucosal cells. Maximum activities were found at the parturition period. Postnatally, four of the activities fell abruptly to the relatively low adult level, when the newborn had sucked (6 hrs of life). The fifth activity, L-alanyl-L-proline dipeptidase, declined successively in the suckling period and reached the adult level first at about 3 weeks *post partum*.

# REFERENCES

- ANDERHALDEN E and Y TERULCHI Studien über die proteolytische Wirkung der Presssaft einiger tierischer Organe sowie des Darmsaftes *Z physiol Chemie* 1906 49 1-14
- ANDERHALDEN R Das Verhalten der Organpeptidasen in verschiedenen Lebensalter *Z Alternsforsch* 1943 4 124-133
- ADAMS E M McFADDEN and E L SMITH Peptidases of erythrocytes I Distribution in man and other species *J biol Chem* 1952 198 663-670
- AGAR W T F J R HIRD and G S SIDHU The active absorption of amino acids by the intestine *J Physiol (Lond)* 1957 121 255-263
- ALVAREZ A and J SAS  $\beta$  galactosidase changes in the developing intestinal tract of the rat *Nature (Lond)* 1961 190 826-827
- AURICCHIO S A RUBINO and G MØRSET Intestinal glycosidase activities in the human embryo fetus and newborn *Pediatrics* 1965 35 944-954
- AURICCHIO S A RUBINO R TOSI G SEMENZA M LANDOLT H KISTLER and A PRADER Disaccharidase activities in human intestinal mucosa *Enzymol biol clin* 1965 5 193-208
- BERGER J and M J JOHNSON Metal activation of peptidases *J Biol Chem* 1959 130 642-654
- BERGMANN M and S J FRUTON Proteolytic enzymes XII Regarding the specificity of aminopeptidase and carboxypeptidase A new type of enzyme in the intestinal tract *J biol Chem* 1937 117 189-202
- BERGMANN M L ZERVAS J S FRUTON F SCHNEIDER and H SCHLEICH On proteolytic enzymes V On the specificity of dipeptidase *J biol Chem* 1935 109 325-346
- BINBLEY F and C TORRES Spectrophotometric assay of peptidase activity *Arch Biochem Biophys* 1960 86 201-203
- BLUM E A I JARMOSCHKEWITSCH and A I JAKOWTSCHIK Die proteolytische Fermente menschlicher Embryonen in den verschiedenen Stadien der Entwicklung *Bull biol med exp USSR* 1936 1 113-114
- BORGSTRÖM B A DAHLQVIST G LUNDH and J SJÖVALL, Studies of intestinal digestion and absorption in the human. *J clin Invest* 1957 36 1521-1536
- BOVEY F A and S S YANARI Pepsin III BOYER P D H LARDY and K MYRBACK (Eds) *The Enzymes* Academic Press New York 1960 4 63-92
- BRONSTEIN H D L J HAEFTNER and O D KOWLESSAR Enzymatic digestion of gliadin The effect of the resultant peptides in adult celiac disease *Clin chim Acta* 1966 14 141-155
- BRYCE C F and H R RABIN The assay and reaction kinetics of leucine aminopeptidase from swine kidney *Biochem J* 1964 90 509-512
- CAMPBELL B J Y C LIN R V DAVIS and E BALLEW The purification and properties of a particulate renal dipeptidase *Biochim biophys Acta (Amst)* 1966 118 371-386

- COHNHEIM O Die Umwandlung des Eiweiss durch die Darmwand *Z physiol Chemie* 1901 33 451-465
- CONNELL, G E G H DIXON and CH S HANES Quantitative chromatographic methods for the study of enzymic transpeptidation reactions *Canad J Biochem* 1955 33 416-427
- CRANE C W and A NEUBERGER The digestion and absorption of protein by normal man *Biochem J* 1960 74 313-323
- DAHLQVIST A The location of carbohydrases in the digestive tract of the pig *Biochem J* 1961 78 281-288
- DAHLQVIST A Rat intestinal dextranase Localization and relation to the other carbohydrases of the digestive tract *Biochem J* 1963 86 72-76
- DAHLQVIST A and T LIDBERG Development of the intestinal disaccharidase and alkaline phosphatase activities in the human foetus *Clin Sci* 1966 30 517-528
- DAHLQVIST A and D L THOMSON The digestion and absorption of lactose by the intact rat *Acta physiol scand* 1964 61 20-33
- DAHLQVIST A B BULL and B E GUSTAFSSON Rat intestinal 6-bromo-2-naphthyl glycosidase and disaccharidase activities 1 Enzymic properties and distribution in the digestive tract of conventional and germ free animals *Arch Biochem. Biophys* 1965 109 150-158
- DAWSON R and E S HOLDSWORTH An investigation into protein digestion with <sup>14</sup>C-labelled protein 1 The general pattern of <sup>14</sup>C incorporation in body tissues and fluids of the rat up to 3 h after feeding *Brit J Nutr* 1962 16 13-25
- DAWSON R and J W G PORTER An investigation into protein digestion with <sup>14</sup>C-labelled protein 2 The transport of <sup>14</sup>C-labelled nitrogenous compounds in the rat and cat *Brit J Nutr* 1962 16 27-38
- DEANBY K G Studien über die proteolytischen Enzyme der Hefe und ihre Beziehung zu der Autolyse *Biochem Z* 1917 81 107-208
- DESNUELLE P Chymotrypsin In BOYER P D H LARDY and K MYRBACK (Eds) *The Enzymes* Academic Press New York 1960 a 4 93-118
- DESNUELLE P Trypsin In BOYER P D H LARDY and K MYRBACK (Eds) *The Enzymes* Academic Press New York 1960 b 4 119-132
- DOELL R G and N KRETCHMER Studies of small intestine during development 1 Distribution and activity of  $\beta$  galactosidase *Biochim biophys Acta (Amst)* 1962 62 353-362
- DOELL R G and N KRETCHMER Invertase in the intestine of the developing rat *Fed Proc* 1963 22 495
- DOELL R G and N KRETCHMER Intestinal invertase Precocious development of activity after injection of hydrocortisone *Science* 1964 143 42-44
- ELLER H Fermentive Spaltung von Dipeptiden *Ark. kemi Mineral Geol* 1907 a 2 nr 31
- ELLER H Fermentive Spaltung von Dipeptiden *Z physiol Chemie* 1907 b 51 213-25
- ELLER H VON and K JOSEPHSON Enzymatische Spaltung von Dipeptiden I *Chem Ber* 1926 a 59 B 226-33
- ELLER H VON and K JOSEPHSON Enzymatische Spaltung von Dipeptiden. II *Z physiol Chemie* 1926 b 157 122-139
- FLEISHER G A Peptidases in human blood I The hydrolysis of glycylglycine and glycyl L-leucine by normal serum *J biol Chem* 1953 205 925-934

- FLEISHER G A, Peptidases in human leucocytes *Ann N Y Acad Sci* 1955 59 1012-1021
- FISHER R B *Protein Metabolism* London Methuen & Co Ltd 1954
- FRAZER, A C The malabsorption syndrome with special reference to the effects of wheat gluten In SOBOTKA H and C H STEWART (Eds) *Advanc clin Chem* 1962 5 69-106
- GAILEY F H and M J JOHNSON Dipeptidases of intestinal mucosa *J biol Chem* 1941 147 921-929
- GLÄSSE, D and H HANSON Einfaches Verfahren zur präparativen Gewinnung hoch gereinigter Leucineaminopeptidase aus Rinderaugenlinsen *Z physiol Chemie* 1962 329 249-256
- GLÄSSE, D and H HANSON Die Wirkungsbedingungen der Glycyl leucin und Alanyl-glycin spaltenden Aktivität der Rinderaugenlinse *Z physiol Chemie* 1963 334 84-94
- GRASSMANN W and H DYCKERHOFF Über die Spezifität der Hefe Peptidasen. *Chem Ber* 1928 61 656-670
- GRASSMANN W and W HEYDE Alkalimetrische Mikrobestimmung der Aminosäuren und Peptide *Z physiol Chemie* 1929 183 32-38
- GRASSMANN W O v SCHOENEBECK and G ALERSBACH Über die enzymatische Spaltbarkeit der Prolinpeptide II *Z physiol Chemie* 1932 210 1-14
- HALLIDAY R The effect of steroid hormones on the absorption of antibody by the young rat *J Endocrin* 1959 18 56-66
- HANSON H and W BLECH Die Lokalisierung von Di- und Amino-peptidaseaktivitäten in den Zellbestandteilkfraktionen von Leber und Niere der Ratte *Z physiol Chemie* 1959 315 191-201
- HASCHEN R J Charakterisierung und Bestimmung der Glycyl L-leucin Dipeptidase der menschlichen Erythrocyten. *Biochem Z* 1961 334 560-568
- HASCHEN R J Charakterisierung und Bestimmung der Glycylglycin Dipeptidase der menschlichen Erythrozyten *Acta biol med germ* 1963 10 468-474
- HILL R L Hydrolysis of proteins In ANFINSEN C B Jr M L ANSON J T Edsall and F M RICHARDS (Eds) *Advanc Protein Chem* 1965 20 37-107
- ISRAHIM J Trypsinogen und Enterokinase beim menschlichen Neugeborenen und Embryo *Biochem Z* 1909 22 24-32
- JAEGER E Über den Eiweissabbau im Fetus *Zbl Gynak* 1907 Nr 35 1060-1062
- JOHNSON F P The development of the mucous membrane of the oesophagus stomach and small intestine in the human embryo *Amer J Anat* 1910 10 521-56
- JOHNSON F P The development of the mucous membrane of the large intestine and vermiform process in the human embryo *Amer J Anat* 1913 14 187-233
- JOHNSON M J and J BERGER The enzymatic properties of peptidases In NORD F F and C H WERKMAN (Eds) *Advanc Enzymol* 1942 2 69-92
- JOHNSON M J G H JOHNSON and W H PETERSON The magnesium activated leucyl peptidase of animal erepsin *J biol Chem* 1936 116 515-56
- JOSEFSSON L Determination of dipeptidase activities *Nature (Lond.)* 1964 204 783-784
- JOSEFSSON L and H SJÖSTRÖM Intestinal Dipeptidases IV Studies on the release and subcellular distribution of intestinal dipeptidases of the mucosal cells of the pig *Acta physiol scand* 1966 67 27-33
- JOSEFSSON L T LINDBERG and A NORDÉN Dipeptidase activity in peroral biopsies of

- small intestinal mucosa XXX Northern Congress Internal Medicine Abo 1966
- KEENE L and E HEWER Digestive enzymes of the human foetus *Lancet* 1969 1 767-769
- KOLDOVSKY O and F CHVETIL Postnatal development of  $\beta$  galactosidase activity in the small intestine of the rat *Biochem J* 1965 94 266-270
- LANGSTEIN L and M SOLDIN Über die Anwesenheit von Erepsin im Darmkanal des Neugeborenen respective Fetus *Jb Kinderheilk* 1908 67 9-12
- LEVENSON S M H ROSEN and H L URJOHN Nature and appearance of protein digestion products in upper mesenteric blood *Proc Soc exp Biol (N Y)* 1959 101 178-180
- LINDERSTRÖM LANG K Titrimetrisk bestemmelse af aminokvælstof *C R Lab Carlsberg* 1927 17 No 4
- LINDERSTRÖM LANG K Über Darmerepsin *Z physiol Chemie* 1929 182 151-174
- LINDERSTRÖM LANG K and H HOLTER Beiträge zur enzymatischen Histochemie I Über die Bestimmungen kleiner enzymatischer Spaltungen *Z physiol Chemie* 1931 201 9-30
- LINDERSTRÖM LANG K and H LANZ JR Studies on enzymatic histochemistry XXIX Dilatometric micro-estimation of peptidase activity *C R Lab Carlsberg* 1938 21 315-338
- LINDERSTRÖM LANG K and M SATO Die Spaltung von Glycylglycin Alanylglycin und Leucylglycin durch Darm und Malzpeptidasen *Z physiol Chemie* 1939 184 83-92
- MAGILL M A R E STEIGER and A J ALLEN XXII Amino acids acyl amino acids dipeptides acyl-dipeptides and derivatives of these compounds I Absorption spectra *Biochem J* 1937 31 188-194
- MASCHMANN E Zur Kenntnis tierischer Peptidasen IV *Biochem Z* 1941 a 309 179-189
- MASCHMANN E Zur Kenntnis tierischer Peptidasen V *Biochem Z* 1941 b 310 28-41
- MATHESON A T and B L TATCHEL A modified Yemm and Cocking ninhydrin reagent for peptidase assay *Canad J Biochem* 1964 42 95-103
- MESSER M CH M ANDERSON and R R W TOWNLEY Peptidase activity of biopsies of the duodenal mucosa of children with and without coeliac disease *Clin chim Acta* 1961 6 768-775
- MESSER M CH M ANDERSON and L HUBBARD Studies on the mechanism of destruction of the toxic action of wheat gluten in coeliac disease by crude papain *Gut* 1964 5 295-303
- MOOG F Developmental adaptations of alkaline phosphatases in the small intestine *Fed Proc* 1962 21 51-56
- MOORE S and W H STEIN Photometric ninhydrin method for use in the chromatography of amino acids *J biol Chem* 1948 176 367-388
- MOORE S and W H STEIN A modified ninhydrin reagent for the photometric determination of amino acids and related compounds *J biol Chem* 1954 211 907-913
- NASSET F S The nutritional significance of endogenous nitrogen secretion in non ruminants In MUNRO H N (Ed) *The role of the gastrointestinal tract in protein metabolism* Blackwell Sci Publ Oxford 1964 83-96
- NELRAATH H Carboxypeptidasen A and B In BOYER P D H LARDY and K MYRBACK (Eds) *The Enzymes* Academic Press New York 1960 4 11-36

- NEWBY H and D H SMYTH The intestinal absorption of some dipeptides *J Physiol* (Lond) 1959 145 48-56
- NEWBY, H and D H SMYTH, Intracellular hydrolysis of dipeptides during intestinal absorption *J Physiol* (Lond) 1960 152 367-380
- NEWBY H and D H SMYTH Cellular mechanisms in intestinal transfer of amino acids *J Physiol* (Lond) 1962 164 5-7-551
- PATTEN B M *Human Embryology* McGraw Hill New York 1953
- PATTERSON E A, H H HSIAO A KEFFEL and S SOROF Studies on dipeptidases and amino peptidases II Zonal electrophoretic separation of rat liver peptidases *J biol Chem* 1965 240 710-716
- PATZELT V, Die feinere Ausbildung des menschlichen Darmes von der fünften Woche bis zur Geburt *Z mikr anat Forsch* 1931 27 269-518
- RADEMAKER W and J B J SOONS A micro-method for the determination of amino acids peptides and proteolytic activity demonstration of the formation of a complex of glycylglycine dipeptidase and cobalt *Biochim biophys Acta* (Amst) 1957 24 209-210
- Report of the Commission on Enzymes of the International Union of Biochemistry* Pergamon Press Ltd Oxford 1961
- ROBINSON G B The hydrolysis of dipeptides by rat intestinal extracts *Biochim. biophys Acta* (Amst) 1960 44 386-387
- ROBINSON G B, The distribution of peptidases in subcellular fractions from the mucosa of the small intestine of the rat *Biochem J* 1963 88 162-168
- ROBINSON G B and B SHAW The hydrolysis of dipeptides by different regions of rat small intestine *Biochem J* 1960 77 351-356
- RUBINO A F ZIMBALATTI and S ALRICCHIO Intestinal disaccharidase activities in adult and suckling rats *Biochim biophys Acta* (Amst) 1964 92 305-311
- SAIDEL L J Ultraviolet absorption spectra of peptides I Compounds containing a single monoalkyl substituted amide link *Arch Biochem Biophys* 1955 54 184-200
- SAIDEL L J A R GOLDFARB and W B KALT False absorption bands in the region of 200-230 m $\mu$  caused by stray radiation in the Beckman spectrophotometer *Science* 1951 113 683-685
- SAIDEL, L J R A GOLDFARB and S WALDMAN The absorption spectra of amino acids in the region two hundred to two hundred and thirty millimicrons *J biol Chem* 195- 197 285-291
- SCHMITT A and G SIEBERT, Optischer Test zur Bestimmung der Peptidaseaktivität *Biochem Z* 1961 334 96-107
- SCHMITT A and G SIEBERT Über die UV Absorption von Dipeptiden aliphatischer Aminosäuren und ihre analytische Bedeutung *Z physiol Chemie* 1964 335 187-202
- SLYKE D D VAN The quantitative determination of aliphatic amino groups II *J biol Chem* 1912 12 275-284
- SLYKE D D VAN and G M MEYER The amino acid nitrogen of the blood Preliminary experiments on protein assimilation *J biol Chem* 1912 12 399-410
- SLYKE D D VAN and G M MEYER The fate of protein digestion products in the body III The absorption of amino acids from the blood by the tissues *J biol Chem* 1913 16 197-212
- SMITH C A *The physiology of the newborn infant* Blackwell Sci Publ Oxford 1959

- SMITH E L. The glycylglycine dipeptidases of skeletal muscle and human uterus *J biol Chem* 1948a 173 571-584
- SMITH E L. Studies on dipeptidases II Some properties of the glycyl L-leucine dipeptidases of animal tissues *J biol Chem* 1948b 176 9-19
- SMITH E L. The specificity of certain peptidases In NORD F F (Ed) *Advan Enzymol* 1951 12 191-257
- SMITH E L. Peptide bond cleavage (Survey) In BOYER P D, H LARDY and K. MYRBACK (Eds) *The Enzymes* Academic Press New York 1960 4 1-10
- SMITH E L. and M. BERGMANN. The peptidases of intestinal mucosa *J biol Chem* 1944 153 627-651
- SMITH E L. and R. L. HILL. Leucine aminopeptidase In BOYER P D, H LARDY and K. MYRBACK (Eds) *The Enzymes* Academic Press New York 1960 4 37-62
- SZAFRAN A. R. SZAFRAN and J. OLESKY. Studies on hydrolases of digestive juices VII. Proteolytic activity of canine intestinal juice *Acta biochem pol* 1962 9 205-214
- SORENSEN S. P. L. Enzymstudien *Biochem Z* 1908 7 45-101
- TACHIBANA T. Physiological investigation of fetus supplementary research of ferments in digestive organs pepton-decomposing ferment in intestinal canal *Jap J Obstet Gynec* 1927 10 40-60
- TOGASAWA Y. and T. KATSUMATA. Effects of metal ions on the glycylglycine dipeptidase II *Bull Jap Soc Sci Fisheries* 1956 21 1070-1075
- TOMAS M. P. F. SOUTER and N. F. MACLAGAN. The spectrophotometric determination of protein at 210 m $\mu$  *Biochem J* 1959 73 167-171
- UGOLEV A. M. Membrane (Contact) Digestion *Physiol Rev* 1965 45 555-595
- UGOLEV, A. M. N. N. IESUTOVA, N. M. TIMOFEEVA and I. N. FEDUSHINA. Location of hydrolysis of certain disaccharides and peptides in the small intestine *Nature (Lond)* 1964 202 807-809
- WALDSCHMIDT LEITZ E. A. K. BALLS and J. WALDSCHMIDT GRASER. Über Dipeptidase und Polypeptidase aus Darm Schleimhaut (XVI Mitteilung zur Spezifität tierischer Proteasen) *Chem Ber* 1929 62B 956-962
- WAGNER H. Bildung und Ausscheidung der proteolytischen Magenfermente beim Fetus und Neugeborenen *Z Kinderheilk* 1958 81 580-588
- WERNER H. Paptic and tryptic capacity of the digestive glands in newborns - A comparison between premature and fullterm infants *Acta Paediat (Stockh)* 1948 35 Suppl 6
- VESCIA A. and A. FIDANZA. Sulle peptidasi della placenta umana. Nota I *Boll Soc Ital Biol sper* 1948 24 823-833
- VESCIA A. and S. TRANIELLO. Effect of metal ions on the specificity of a peptidase isolated from swine kidney *Ital J Biochem* 1964 13 374-385
- WETLAUFER D. B. Ultraviolet spectra of proteins and amino acids In ANFENSEN C. B. JR, M. L. ANSON, K. BAILEY and J. T. EDSALL (Eds) *Advan Protein Chem* 1962 17 303-390
- WIGGANS D. E. and J. M. JOHNSTON. The absorption of peptides *Biochim biophys Acta (Amst)* 1959 32 69-73
- WILCOX H. G. and M. FRIED. Studies on rat liver glycylglycine dipeptidase *Biochem J* 1963 87 192-199
- ZOCH E. Über die Peptidasen Aktivität der Menschlichen Placenta *Enzymologia* 1965 28 3 5-332











**ACTA PHYSIOLOGICA SCANDINAVICA**  
**VOL. 69 SUPPLEMENTUM 286**

**HISTOCHEMISTRY OF THE CILIARY  
GANGLION OF THE RAT AND THE EFFECT  
OF PRE- AND POSTGANGLIONIC  
NERVE DIVISION**

**BY**  
**KAUKO T HUIKURI**

**HELSINKI 1966**



ACTA PHYSIOLOGICA SCANDINAVICA  
VOL 69 SUPPLEMENTUM 286

FROM THE DEPARTMENT OF ANATOMY UNIVERSITY OF HELSINKI  
SILTAVUORENPENGER HELSINKI FINLAND

HISTOCHEMISTRY OF THE CILIARY  
GANGLION OF THE RAT AND THE EFFECT  
OF PRE- AND POSTGANGLIONIC  
NERVE DIVISION

BY  
KAUKO T HUIKURI

HELSINKI 1966



## PREFACE

The theme of the present study was proposed in 1964 by Professor Olavi Eranko M.D. Ph.D. Head of the Department of Anatomy University of Helsinki. This work is part of a research project on the histochemistry of the nervous tissue directed by him.

It is a pleasure to express my deep gratitude to Professor Eranko for his supervision, encouragement and expert criticism during all stages of the work.

I am indebted to Professor Seppo Talanti D.V.M. M.L. Head of the Department of Anatomy, College of Veterinary Medicine, Helsinki, for his constructive criticism of the manuscript.

I wish to express my sincere thanks to Dr. Aulikki Kokko M.D. for many valuable discussions and for her guidance under which the present work was done. I am grateful to Dr. Martti Ikonen M.D. for revision of a part of the manuscript.

I owe special thanks to my colleagues in the Department of Anatomy for their pleasant co-operation and helpful attitude during this work.

My thanks are furthermore due to Miss Paula Alahaivala and Mrs. Pirkko Alanko for their excellent technical assistance and to Miss Helmi Palomaki for help in obtaining the literature.

Helsinki August 1966

*Kauko T. Huikuri*





Alkaline Phosphatase  
Adenosine Triphosphatase  
Effect of Denervation  
Preganglionic Denervation  
Postganglionic Denervation

#### Methods

#### Results

Normal Ganglion  
Acid Phosphatase  
Alkaline Phosphatase  
Adenosine Triphosphatase  
Preganglionic Denervation  
Postganglionic Denervation  
Sympathectomy

#### Discussion

Acid Phosphatase  
Alkaline Phosphatase  
Adenosine Triphosphatase

### III OXIDATIVE ENZYMES

#### Earlier Investigations

Normal Nervous Tissue

Dehydrogenases

Oxidases

Intracellular Distribution of Oxidative Enzymes

Effect of Denervation

Preganglionic Denervation

Postganglionic Denervation

#### Methods

Dehydrogenases

Oxidases

#### Results

Normal Ganglion

Dehydrogenases

Oxidases

Preganglionic Denervation

Postganglionic Denervation

Sympathectomy

#### Discussion

Dehydrogenases

Oxidases

### IV CATECHOLAMINES

Earlier Investigations

Method

Results

Discussion

SUMMARY

REFERENCES



## INTRODUCTION

Adamuk (1870) was the first to point out that the ciliary ganglion of mammals was a special type of ganglion, neither sympathetic nor sensory. Since then however the nature of this ganglion has been a very controversial subject (Schwalbe 1879 Retzius 1894).

The ciliary ganglion cells have been divided into eight categories (Pines 1927) a classification which is of histological interest but difficult to interpret functionally. The majority of the neurones of the mammalian ciliary ganglion are multipolar although some bipolar nerve cells can also be found. The neurones which are all of nearly the same size are surrounded by a mantle of satellite cells under which most of the numerous synapses are located. The dendritic processes are short forming a plexus almost exclusively under the satellite cell capsule. The axon is usually long (Retzius 1894 Slavich 1932).

Enzyme histochemistry supported by modern microbiocchemistry has thrown light on the character and function of the ciliary ganglion cells. Investigations made by these methods have established that the mammalian ciliary ganglion cells are purely cholinergic that is to say that acetylcholine is the transmitter substance in the synapses of this ganglion (Koelle 1955 Jacobowitz 1959 Koelle and Koelle 1959 Taxi 1961). On the other hand a few pharmacological studies have indicated that some kind of adrenergic synaptic transmission takes place in this ganglion as well (Tum Suden *et al* 1951 1952 Perry and Talesnik 1953 Perry 1957).

However relatively little work has been done towards elucidating the enzyme histochemistry of the nervous system especially of its parasympathetic part. Except in the case of the cholinesterases extremely few investigations concerning the enzymes in the parasympathetic nervous tissue have been made. There are still fewer studies concerning the effect of experimental procedures e.g. nerve division on the behaviour of enzyme activities. A systematic investigation of hydrolysing and oxidative enzymes is totally lacking. A previous study made in this laboratory on the superior cervical ganglion of the rat (Harkonen 1964) has proved the value of such an approach.

The aim of the present study has been in fact to elucidate the histochemistry of enzymes such as carborylic esterases phosphatases and oxidative enzymes in the ciliary ganglion of the rat under normal conditions and at varying periods after pre and postganglionic denervation or after extirpation of the superior cervical ganglion. Catecholamines have also been studied in the normal ciliary ganglion of the rat.



## GENERAL MATERIAL AND METHODS

### *Normal Material*

The normal material comprised about 200 male and female rats of the Sprague-Dawley strain. Their ages ranged from 3 to 12 months. The animals were killed by decapitation under light ether anaesthesia. Both ciliary ganglia were immediately removed by the intraorbital route as follows. After decapitation the head of the rat was skinned. The zygomatic arches were cut through and the lateral part of the Harderian gland excised. The ganglia were then removed with a small portion of the oculomotor nerve. The whole procedure of removing both ganglia took about 5 minutes.

For histochemical studies formalin-fixed, fresh-frozen and freeze-dried ganglia were used. Formalin fixation was carried out at 4°C with formal-calcium (Eranko 1959) for 6 hours. Thereafter the ganglia were embedded in 10% gelatin replaced in formal-calcium and left overnight. After fixation the specimens were cut with a freezing microtome at 8 $\mu$ . These sections were stained free floating. For the fresh and postfix fixed section the newly resected ganglia were mounted in 10% gelatin on a steel tissue holder and frozen in liquid nitrogen. The specimens were sectioned with a cryostat microtome at 8 $\mu$ . These sections were mounted on clean coverslips and dried at room temperature for 10 minutes. The fresh sections were then preincubated or incubated. Some of the cryostat sections were also postfix fixed in formal-calcium or in acetone in a refrigerator for 15 minutes. The freeze-drying procedure is described in connexion with the catecholamines (Chapter IV).

### *Experimental Procedures*

Denervation was performed under ether anaesthesia with the aid of a dissecting microscope. The left ciliary ganglion was approached by an extracranial route. A small skin incision was made perpendicular to the zygomatic arch and a small part of this was resected. The lateral part of the Harderian gland was removed or partly drawn out from the orbit. The ganglion was denervated in two ways: (1) Preganglionically by dissecting the oculomotor nerve about 12 mm centrally from the ganglion (decentralization) and (2) postganglionically by cutting all postganglionic nerve fibres (axotomy). The criterion of the success of the operation was maximal dilatation of the lateral pupil. Decrease in size of the pupil was used as a criterion of functional recovery.

The effect of removal of the left superior cervical ganglion (sympathectomy) on the ciliary ganglion was also studied. Therefore a longitudinal incision was made in the neck of the rat and the ganglion was approached bluntly and excised. As a criterion of the success of the operation Horner's syndrome in the ipsilateral part of the head was used.

In every experiment the contralateral unoperated ganglion was used as a control. It was always like the ciliary ganglion of an untreated animal.

The postoperative recovery period varied from 1 to 90 days. The number of rats killed at different times after the operation is seen in Table I.

TABLE I

*Ciliary ganglia studied at different times after  
three types of nerve division*

Days after nerve division	Number of rats		
	Decentralization	Axotomy	Sympathectomy
1	6	11	
3	6	12	
5	12	12	10
10	12	14	10
15	12	15	
30	13	15	10
60	13	15	
90	15	16	

# I CARBOXYLIC ESTERASES

## EARLIER INVESTIGATIONS

### NORMAL NERVOUS TISSUE

#### *Acetylcholinesterase*

Acetylcholinesterase (AChE) or true or specific cholinesterase which is known to play an important role in the transmission of nervous impulses is the most widely studied enzyme belonging to the group of carboxylic esterases. According to Koelle's hypothesis (1962) acetylcholine (ACh) is the original transmitter substance in cholinergic and noncholinergic neurones. This view is supported by studies revealing the presence of AChE which hydrolyses ACh in all nervous tissues (Nachmansohn 1959).

*Ciliary Ganglion* Intense AChE activity has been reported in the ciliary ganglion of many species. The positive AChE reaction obtained when acetylthiocholine is used as substrate in combination with various specific inhibitors has been described in the cytoplasm of the ciliary ganglion cells of the cat (Koelle 1950, 1951, 1955; Fukuda and Koelle 1959; Koelle and Koelle 1959; McIsaac and Koelle 1959; Cauna *et al.* 1961; Taxi 1961; Fredricsson and Sjöqvist 1962), the rabbit, the rhesus monkey (Koelle 1955), the hen (Szentagothai *et al.* 1955), the chicken (Taxi 1961), the dog, the horse and man (Okinaka *et al.* 1963).

Two morphologically and enzymatically distinct types of neurones were reported in the ciliary ganglion of the cat (Whittenridge 1937; Koelle 1950). However, Koelle's later (1951, 1955) investigations and those made by other authors (Fukuda and Koelle 1959; Koelle and Koelle 1959; McIsaac and Koelle 1959; Cauna *et al.* 1961; Taxi 1961; Fredricsson and Sjöqvist 1962; Okinaka *et al.* 1963) have established that all the ganglion cells in the mammals studied exhibit an intense AChE activity. The high activity was uniformly distributed in the ganglion cell cytoplasm and in the cell processes which seemed to be mainly subcapsular dendrites enveloping the cell body. In birds in which the ciliary ganglion cells are unipolar (Lenhossek 1911) the cytoplasmic AChE reactivity was moderate in all neurones but the perineuronal activity was exclusively concentrated in one end of the ganglion cell.

The high synaptic AChE activity in the ciliary ganglion has been emphasized both in mammals (Koelle 1951; Fukuda and Koelle 1959; Koelle and Koelle 1959; Cauna *et al.* 1961; Taxi 1961; Fredricsson and Sjöqvist 1962) and in birds (Szentagothai *et al.* 1955; Szentagothai 1957; Taxi 1961). In mammals the activity is distributed in both the pre- and postganglionic part of the synapse although the greater part is supposed to be localized postsynaptically (Koelle 1951). In birds the activity is mainly situated



in the calyciform (De Lorenzo 1960 Takahashi and Hama 1965 1965 a) presynaptic part of the synapse although Szentagothai *et al* (1955) found some activity postsynaptically as well

Some nerve fibres also show the AChE activity in the ciliary ganglion (Koelle 1951 1955 Koelle and Koelle 1959 Taxi 1961 Okinaka *et al* 1963) Most of the active fibres are postganglionic while the preganglionic fibres stain very faintly (Koelle 1951 Gerebtzoff 1959 Taxi 1961) although Okinaka *et al* (1963) have demonstrated a very high AChE activity in the oculomotor nerve there being no difference between the autonomic and somatic fibres The AChE positive fibres are less numerous in the ciliary ganglion than in other ganglia (Koelle 1951) However even after prolonged incubation Cauna *et al* (1961) were not able to demonstrate any AChE positive nerve fibres in the ciliary ganglion of the cat

Biochemical studies using a microgasometric technique demonstrated a high AChE activity in all the isolated ciliary ganglion cells of the rat only the anterior horn cells of the spinal cord showed a slightly higher activity The AChE concentration varied very little between the individual cells in contrast with the sympathetic and sensory neurones in which great variation in the activity of this enzyme existed between different cells (Giacobini 1959 1959 a 1960)

*Other Nervous Tissues* In the sympathetic ganglion cells the AChE activity varies considerably between individual neurones as demonstrated histo- and biochemically A minority of the nerve cells possess a high enzyme activity the rest of the ganglion cells are moderately or lightly stained (Koelle 1951 1955 Giacobini 1956 1957 Snell 1958 Holmstedt and Sjoqvist 1959 Koelle and Koelle 1959 Cauna *et al* 1961 Taxi 1961 Harkonen 1964) This is also the case with sensory nerve cells However some sensory neurones are devoid of any AChE activity (Koelle 1951 1955 Giacobini 1959 a Cauna and Nisk 1963 Esila 1963 Tewari and Bourne 1963 Kokko 1965) while motor nerve cells usually exhibit a high AChE activity (Nachmansohn and Hoff 1944 Koelle 1951 1955 Giacobini and Holmstedt 1958 Schwarzacher 1958 Giacobini 1960 Soderholm 1965) Giacobini (1956) has studied histochemically isolated sympathetic and spinal ganglion cells and motor neurones of the rat and noticed the same distribution of AChE activity as described above

A high AChE activity has been demonstrated in the preganglionic axons and their terminals in the sympathetic ganglia (Koelle 1951 1955 Koelle and Koelle 1959 Cauna *et al* 1961 Taxi 1961 Harkonen 1964) while in motor neurones the enzyme activity is high in the pre- and postganglionic axons and synaptic structures (Schwarzacher 1958 Soderholm 1965)

*Intracellular Localization* The distribution of histochemically demonstrable cytoplasmic AChE activity corresponds to that of the Nissl substance (Fukuda 1959 Fukuda and Koelle 1959) which in turn probably represents the granular endoplasmic reticulum (Palay and Palade 1955) where AChE may be synthesized and thereafter transported to the surface of the cell body and to its processes (Fukuda and Koelle 1959) Biochemical studies based on differential centrifugation have shown this view to be correct AChE being mainly associated with the membranous structures of the microsomal fraction (Nathan and Aprison 1955 Aldridge and Johnson 1959 Hanzon and Toschi 1959 Holmstedt and Toschi 1959 Toschi 1959) Later investigations into the subcellular distribution of AChE ACh and choline acetylase (ChA) have demonstrated that AChE is localized mainly in the membranous part of the nerve endings ACh in

the synaptic vesicles and ChA partly in the synaptic vesicles partly in the free cytoplasm (Whittaker 1959 Gray and Whittaker 1960 De Robertis *et al* 1961 1962 1963 Rodriguez De Lores Arnaiz 1964 Whittaker *et al* 1964 Whittaker and Sheridan 1965). In electron microscopic studies the AChE activity has been observed to be localized in the endoplasmic reticulum in the pre and postsynaptic membranes (Torack and Barnett 1962 Lewis and Shute 1964 1965 Lewis *et al* 1965 Koelle and Foroglou Kerameos 1965 Shute and Lewis 1966) and in the axonal membrane (Lewis and Shute 1965 a Shute and Lewis 1966).

### *Non specific Cholinesterase*

Non specific cholinesterase (ns ChE) in the ciliary ganglion has been reported to be localized almost exclusively to the satellite cells the Schwann cells and the nerve fibres (Koelle 1951 1955 Cauna *et al* 1961 Taxi 1961). The animals studied were the cat the rabbit the monkey and the hen. The ganglion cell cytoplasm was totally devoid of histochemically demonstrable activity. These results are supported by biochemical studies on rat ciliary ganglion cells (Giacobini 1959 1960) Caslik and Savay (1954) demonstrated histochemically an intense ns ChE activity in every ciliary ganglion cell of the rat while glial cells and nerve fibres were less strongly stained. However the method used by them probably revealed the non specific esterase activity as well.

A similar distribution of ns ChE activity has been seen in the sympathetic and sensory ganglion cells of birds and mammals excluding the rat (Koelle 1951 1955 Giacobini 1956 1960 Holmstedt 1957 Cauna *et al* 1961 Taxi 1961 Cauna and Naik 1963). By contrast some sympathetic and sensory neurones of the rat exhibit a moderate ns ChE activity (Giacobini 1959 Cauna *et al* 1961 Taxi 1961 Harkonen 1964 Hokko 1965) while the motor neurones show no ns ChE activity at all (Koelle 1951 1955 Schwarzacher 1958 Giacobini 1959 1960 Soderholm 1965).

Intracellularly ns ChE is mainly distributed in the nuclear microsome and supernatant fractions (Aldridge and Johnson 1959). Ultrastructurally ns ChE is mainly localized in the endoplasmic reticulum in the hypoglossal neurones of the rat (Lewis and Shute 1965 a).

### *Non specific Esterases*

The division of the non specific esterases (ns Es) into E 600-sensitive non specific esterase (Fs ns E) and E 600-resistant non specific esterase (Er ns E) according to whether or not they are inhibited by diethyl p-nitrophenyl phosphate (E 600) has seldom been used in the previous investigations which makes their interpretation difficult.

Savay *et al* (1953) using  $\beta$ -naphthyl acetate as substrate and acetone fixed paraffin sections described an intense ns F activity in nearly all the ciliary ganglion cells and cell processes of the cat. The glial cells and nerve fibre plexuses around the neurones were moderately positive while the postganglionic nerve fibres were negative.

The ns E activity varies between individual nerve cells in the sympathetic spinal and other ganglia of the rabbit dog and cat (Savay *et al* 1953). In the rat the E-s ns E activity is mainly localized in the cytoplasm of the sympathetic (Harkonen 1964) spinal

in the calyxiform (De Lorenzo 1960 Takahashi and Hama 1965 1965 a) presynaptic part of the synapse although Szentagothai *et al* (1955) found some activity postsynaptically as well

Some nerve fibres also show the AChE activity in the ciliary ganglion (Koelle 1951 1955 Koelle and Koelle 1959 Taxi 1961 Okinaka *et al* 1963) Most of the active fibres are postganglionic while the preganglionic fibres stain very faintly (Koelle 1951 Gerebtzoff 1959 Taxi 1961) although Okinaka *et al* (1963) have demonstrated a very high AChE activity in the oculomotor nerve there being no difference between the autonomic and somatic fibres The AChE positive fibres are less numerous in the ciliary ganglion than in other ganglia (Koelle 1951) However even after prolonged incubation Cauna *et al* (1961) were not able to demonstrate any AChE positive nerve fibres in the ciliary ganglion of the cat

Biochemical studies using a microgasometric technique demonstrated a high AChE activity in all the isolated ciliary ganglion cells of the rat only the anterior horn cells of the spinal cord showed a slightly higher activity The AChE concentration varied very little between the individual cells in contrast with the sympathetic and sensory neurones in which great variation in the activity of this enzyme existed between different cells (Giacobini 1959 1959 a 1960)

*Other Nervous Tissues* In the sympathetic ganglion cells the AChE activity varies considerably between individual neurones as demonstrated histo- and biochemically A minority of the nerve cells possess a high enzyme activity the rest of the ganglion cells are moderately or lightly stained (Koelle 1951 1955 Giacobini 1956 1957 Snell 1958 Holmstedt and Sjoqvist 1959 Koelle and Koelle 1959 Cauna *et al* 1961 Taxi 1961 Harkonen 1964) This is also the case with sensory nerve cells However some sensory neurones are devoid of any AChE activity (Koelle 1951 1955 Giacobini 1959 a Cauna and Naik 1963 Esila 1963 Tewari and Bourne 1963 Kokko 1965) while motor nerve cells usually exhibit a high AChE activity (Nachmansohn and Hoff 1944 Koelle 1951 1955 Giacobini and Holmstedt 1958 Schwarzacher 1958 Giacobini 1960 Soderholm 1965) Giacobini (1956) has studied histochemically isolated sympathetic and spinal ganglion cells and motor neurones of the rat and noticed the same distribution of AChE activity as described above

A high AChE activity has been demonstrated in the preganglionic axons and their terminals in the sympathetic ganglia (Koelle 1951 1955 Koelle and Koelle 1959 Cauna *et al* 1961 Taxi 1961 Harkonen 1964) while in motor neurones the enzyme activity is high in the pre- and postganglionic axons and synaptic structures (Schwarzacher 1958 Soderholm 1965)

*Intracellular Localisation* The distribution of histochemically demonstrable cytoplasmic AChE activity corresponds to that of the Nissl substance (Fukuda 1959 Fukuda and Koelle 1959) which in turn probably represents the granular endoplasmic reticulum (Palay and Palade 1955) where AChE may be synthesized and thereafter transported to the surface of the cell body and to its processes (Fukuda and Koelle 1959) Biochemical studies based on differential centrifugation have shown this view to be correct AChE being mainly associated with the membranous structures of the microsomal fraction (Nathan and Aprison 1955 Aldridge and Johnson 1959 Hanzon and Toschi 1959 Holmstedt and Toschi 1959 Toschi 1959) Later investigations into the subcellular distribution of AChE ACh and choline acetylase (ChA) have demonstrated that AChE is localized mainly in the membranous part of the nerve endings ACh in

the synaptic vesicles and ChA partly in the synaptic vesicles partly in the free cytoplasm (Whittaker 1959 Gray and Whittaker 1960 De Robertis *et al* 1961 1962 1963 Rodriguez De Lores Arnaiz 1964 Whittaker *et al* 1964 Whittaker and Sheridan 1965). In electron microscopic studies the AChE activity has been observed to be localized in the endoplasmic reticulum in the pre and postsynaptic membranes (Torack and Barnett 1962 Lewis and Shute 1964 1965 Lewis *et al* 1965 Koelle and Foroglou Kerameos 1965 Shute and Lewis 1966) and in the axonal membrane (Lewis and Shute 1965 a Shute and Lewis 1966).

### *Non specific Cholinesterase*

Non specific cholinesterase (ns ChE) in the ciliary ganglion has been reported to be localized almost exclusively to the satellite cells the Schwann cells and the nerve fibres (Koelle 1951 1955 Cauna *et al* 1961 Taxi 1961). The animals studied were the cat the rabbit the monkey and the hen. The ganglion cell cytoplasm was totally devoid of histochemically demonstrable activity. These results are supported by biochemical studies on rat ciliary ganglion cells (Giacobini 1959 1960) Csulik and Savay (1954) demonstrated histochemically an intense ns ChE activity in every ciliary ganglion cell of the rat while glial cells and nerve fibres were less strongly stained. However the method used by them probably revealed the non specific esterase activity as well.

A similar distribution of ns ChE activity has been seen in the sympathetic and sensory ganglion cells of bird and mammals excluding the rat (Koelle 1951 1955 Giacobini 1956 1960 Holmstedt 1957 Cauna *et al* 1961 Taxi 1961 Cauna and Naik 1963). By contrast some sympathetic and sensory neurones of the rat exhibit a moderate ns ChE activity (Giacobini 1959 Cauna *et al* 1961 Taxi 1961 Harkonen 1964 Kokko 1965) while the motor neurones show no ns ChE activity at all (Koelle 1951 1955 Schwarzscher 1958 Giacobini 1959 1960 Sodetholm 1965).

Intracellularly ns ChE is mainly distributed in the nuclear microsomal and supernatant fractions (Aldridge and Johnson 1959). Ultrastructurally ns ChE is mainly localized in the endoplasmic reticulum in the hypoglossal neurones of the rat (Lewis and Shute 1965 a).

### *Non specific Esterases*

The division of the non specific esterases (ns Es) into E 600 sensitive non specific esterase (Fs ns E) and E 600 resistant non specific esterase (Er ns E) according to whether or not they are inhibited by diethyl p nitrophenyl phosphate (E 600) has seldom been used in the previous investigations which makes their interpretation difficult.

Savay *et al* (1953) using  $\beta$ -naphthyl acetate as substrate and acetone fixed paraffin sections described an intense ns E activity in nearly all the ciliary ganglion cells and cell processes of the cat. The glial cells and nerve fibre plexuses around the neurones were moderately positive while the postganglionic nerve fibres were negative.

The ns E activity varies between individual nerve cells in the sympathetic spinal and other ganglia of the rabbit dog and cat (Savay *et al* 1953). In the rat the Fs ns E activity is mainly localized in the cytoplasm of the sympathetic (Harkonen 1964) spinal

(Thomas 1963 Kokko 1965) and other ganglion cells (Fisher and Sutherland 1965 1965 a) and the activity varies moderately between individual neurones while in the motor nerve cells this variation is less distinct (Soderholm 1965) The activity of Er ns E is much weaker than that of Es ns E and it is mainly localized in the cytoplasmic granules in the nerve cells (Harkonen 1964 Fisher and Sutherland 1965 a Kokko 1965 Soderholm 1965)

Biochemical studies have demonstrated that the mitochondrial and microsomal fractions possess the highest ns E activity in the brain tissue (Aldridge and Johnson 1959 Sellinger and De Balbian Verster 1962) Electron microscopic studies have revealed that Es ns E is localized in the nuclear membrane endoplasmic reticulum and Golgi apparatus its distribution resembling that of ns ChE while Er ns E is mostly situated in dense bodies in the neurones and neuroglial cells of the rat brain tissue (Torack and Barnett 1962)

## EFFECT OF DENERVATION

### *Preganglionic Denervation*

*Acetylcholinesterase* : Division of the preganglionic nerve trunk of the ciliary ganglion in the hen and the chick was reported to result in the disappearance of AChE activity in the preganglionic synaptic structures the postganglionic synaptic activity remaining intact (Szentágothai *et al* 1955 Taxi 1961) The change in enzyme activity was seen after 2 days and reached a maximum within a week after the operation In the ciliary ganglion of the cat a considerable decrease in the number of active intraganglionic nerve fibres was observed after decentralization In addition to this the enzyme activity at the periphery of the neurones was reported by Koelle and Koelle (1959) to be much decreased while Taxi (1961) did not find any marked change in the reactivity of this synaptic zone However some formerly active ganglion cells lost much of their reactivity which may have been due to operational interference with the post ganglionic fibres (Taxi 1961)

Decentralization also brings about the disappearance of AChE activity from the preganglionic nerve fibres and their terminals in the sympathetic ganglia of the rat (Brown 1958 Taxi 1961 Harkonen 1964) and the cat (Koelle 1955 Snell 1958 Koelle and Koelle 1959 Fredricsson and Sjoqvist 1962 Holmstedt *et al* 1963)

Biochemical studies have confirmed that after decentralization a definite decrease in the enzyme activity occurs in the sympathetic ganglia of the rat (Dhar 1958 Harkonen 1964) and the cat (von Brucke 1937 Sawyer and Hollinshead 1945 Holmstedt *et al* 1963 Gromadzki and Koelle 1965)

*Non specific Cholinesterase* Taxi (1961) found no change in the ns ChE activity in the ciliary ganglion of the cat after decentralization and the result was the same with the superior cervical ganglion of the rat and the cat (Taxi 1961 Fredricsson and Sjoqvist 1962) Other authors on the contrary have demonstrated a marked decrease in the ns ChE activity in the sympathetic ganglia of the rat and the cat as studied histo- and biochemically (Dhar 1958 Snell 1958 Harkonen 1964)

*Non specific Esterases* Neither  $E_s$  ns  $E$  nor  $E_r$  ns  $E$  showed any histo- or biochemically demonstrable changes in activity in the superior cervical ganglion of the rat after preganglionic nerve division (Harkonen 1964). No report is available concerning the ciliary ganglion and ns  $E_s$  after this type of operation.

### *Postganglionic Denervation*

*Acetylcholinesterase* Szentágothai *et al* (1955) did not notice any change in the AChE activity in the ciliary ganglion of the hen after axotomy while Taxi (1961) described a distinct decrease of the enzyme activity in the ciliary ganglion cells of the cat and the chick. In the former species nearly all the AChE activity disappeared from the ganglion cell cytoplasm in 10 days while the perineuronal synaptic activity was affected much less than after decentralization.

After axotomy a great diminution in histochemically demonstrable AChE activity in the neuronal cytoplasm, synapses and postganglionic nerve fibres of the sympathetic ganglia has been observed in the rat (Brown 1958, Taxi 1961, Harkonen 1964, Eranku and Harkonen 1965) and the cat (Fredricsson and Sjoqvist 1962, Gromadzki and Koelle 1965). Similar changes have been found in the motor neurones of the rat (Schwarzacher 1958, Soderholm 1965) and the toad (Chacko and Cerf 1960). In addition Harkonen (1964) and Soderholm (1965) have reported a loss of AChE from the preganglionic nerve fibres and synaptic terminals after axotomy.

These findings have been confirmed by biochemical studies concerning the AChE content of the axotomized sympathetic ganglia of the rat (Dhar 1958, Harkonen 1964) and the cat (Sawyer and Hollinshead 1945, Brown *et al* 1952, McLennan 1954, Gromadzki and Koelle 1965). Lewis and Shute (1965a) have reported a great decrease of AChE activity in the hypoglossal neurones of the rat after axotomy as studied with the electron microscope. Many nerve fibres proximal to the cut exhibited foldings of the axonal membrane with enhanced enzyme activity while in the distal segment there was complete loss of enzyme activity.

*Non specific Cholinesterase* A loss of this enzyme from the neuronal cytoplasm and a marked decrease of activity in the glia were demonstrated by Harkonen (1964) in the axotomized superior cervical ganglion of the rat whereas Taxi (1961) and Fredricsson and Sjoqvist (1962) failed to observe any such change after axotomy in the enzyme activity of the sympathetic ganglia of the rat and the cat respectively. In the central nervous system in which the activity is restricted almost exclusively to the capillaries ns ChE has not been observed to undergo any change after axotomy (Schwarzacher 1958, Soderholm 1965).

Biochemical determinations have verified that a definite decrease in ns ChE activity takes place in the sympathetic ganglia of the rat (Dhar 1958, Harkonen 1964) and the cat (Sawyer and Hollinshead 1945) after axotomy. In the latter case almost total disappearance of the activity of the enzyme was observed. Electron microscopically it was seen that almost all the ns ChE activity disappeared from the cytoplasm of the neurones of the hypoglossal nuclei of the rat after axotomy (Lewis and Shute 1965a).

*Non specific Esterases* The  $E_s$  ns  $E$  activity in the sympathetic ganglion cells of the rat diminished after axotomy but there was no such change in the  $E_r$  ns  $E$  activity (Harkonen 1964). In the anterior horn cells of the spinal cord of the rat both these

enzymes showed a markedly decreased activity after postganglionic denervation (Soderholm 1965) while in the spinal cord of the guinea pig only a slight decrease of  $n_sE$  activity was observed with naphthol AS-acetate as substrate after sciatic neurotomy (Kumamoto and Bourne 1963). After an initial increase in the nodose ganglion and dorsal vagal nucleus of the monkey following cervical vagotomy a fall in the organophosphate resistant indoxyl esterase activity to below the normal level was described by Fisher and Sutherland (1963a).

Biochemically diminished activity of both  $E_s$  and  $E_r$  was demonstrated in the sympathetic ganglion of the rat after axotomy although the latter enzyme had lost only about 14% of its activity (Harkonen 1964).

### *Sympathectomy*

Çallik and Koelle (1963) described a slight reduction (15–20%) in the number of AChE positive nerve fibres in the rat iris after extirpation of the ipsilateral superior cervical ganglion while Ehinger and Falck (1965) did not observe any change in the number or staining intensity of the AChE positive fibres in the rat iris after removal of the cervical sympathetic chain. There is no report available about the effect of these operations on the enzyme activity of the ciliary ganglion.

## METHODS

### *Cholinesterases*

Gomori's (1952) modification of Koelle's (1951) method was used for the demonstration of cholinesterase. Sections were preincubated in the stock solution with or without inhibitor for 30 minutes at 37°C and thereafter incubated for 2–6 hours in the same medium to which the substrate acetyl or butyrylthiocholine iodide was added.

To differentiate between acetylcholinesterase and non specific cholinesterase (Augustinsson and Nachmansohn 1949) the following inhibitors were used: 1.5 bis-(4-allyl dimethylammoniumphenyl) pentan-3-one-diiodide (284 C 51) in a concentration of 10  $\mu$ M to inhibit AChE selectively (Austin and Berry 1953; Bayliss and Todrick 1956) and tetra isopropylpyrophosphoramide (iso-OMPA) in a concentration of 10  $\mu$ M to inhibit  $n_sChE$  selectively (Aldridge 1953; Austin and Berry 1953; Bayliss and Todrick 1956; Diegenbach 1965). The concentrations of these inhibitors were checked before the routine procedures.

Control sections were incubated without the substrate or by using both 10  $\mu$ M iso-OMPA and 10  $\mu$ M 284 C 51 together or 10  $\mu$ M physostigmine (evimeline) alone to inhibit ChEs (Augustinsson 1948; Gomori 1952; Pearse 1960). These controls were always totally negative.

### Non specific Esterases

Non specific esterases can be divided into two groups as in previous studies made in this laboratory (1) E 600-sensitive non specific esterase and (2) E 600-resistant non specific esterase according to their behaviour towards E 600 as inhibitor (Aldridge 1953 Eranko *et al* 1962 1964 Harkonen 1964 Kokko 1965 Soderholm 1965) Es ns E which is eserine resistant is selectively demonstrated in fresh sections and Es ns E in fixed sections (Eranko *et al* 1964 Harkonen 1964 Kokko 1965 Soderholm 1965)

The sections were preincubated with or without any inhibitor in a buffer solution at the same pH as that of the incubation medium for 30 minutes before incubation

In addition to the above mentioned inhibitors eserine in a concentration of 10<sup>-4</sup> M was used to inhibit both ChEs (Richter and Croft 1942 Augustinsson 1948 Summer and Myrback 1950 Aldridge 1953 a) E 600 (Parovon Mintacol) 10 M was used to differentiate between Es ns E and Es ns E (Aldridge and Davison 1952 Aldridge 1954 Hobbiger 1957 Eranko *et al* 1964) AChE was inhibited with 10<sup>-4</sup> M 284 C 51 and ns ChE with 10<sup>-4</sup> iso-OMPA

The substrates used were  $\alpha$ -naphthyl acetate and butyrate 4-chloro-5-bromindoxyl acetate and naphthol ASD acetate

**$\alpha$ -Naphthyl Acetate and Butyrate** The method described by Nachlas and Seligman (1949) was used as later modified by Pearse (1960) and Eranko *et al* (1962) The incubation time was 35 minutes with  $\alpha$ -naphthyl acetate and 25 minutes with  $\alpha$ -naphthyl butyrate Blue RR Salt was used as coupling agent

**4-Chloro-5-bromindoxyl Acetate** The method used was that of Holt (1952) and Holt and Withers (1952) The incubation time was 75 minutes for fresh and postfixed sections and 45 minutes for fixed sections

**Naphthol ASD Acetate** Planteyds (1961) modification of Gossner's method (1958) was employed except that Blue RR Salt was used as coupler The incubation time was 2 hours for fresh and postfixed sections and 35 minutes for fixed sections

The control for all these reactions were made by incubating the sections without either the substrate or the coupling agent These controls were always negative



## RESULTS

## NORMAL GANGLION

In the presentation of the results the carboxylic esterases are classified according to the principle adopted in this laboratory (Eranko *et al* 1964 Harkonen 1964 Kokko 1965 Soderholm 1965) The esterases are divided into two main groups ChEs and ns Es ChEs are subdivided into AChE and ns ChE using *iso* OMPA and 284 C 51 as specific inhibitors Ns Es are grouped into Es ns E and Er ns E using E 600 as differentiating inhibitor

The most reliable methods for demonstrating the four groups of carboxylic esterases are presented in the following scheme

Enzyme	Type of section	Substrate	Inhibitor
AChE	Fresh postfixed fixed	Acetylthiocholine iodide $6.9 \times 10^{-5} \text{M}$	<i>iso</i> -OMPA $10^{-6} \text{M}$
Ns ChE	Fresh postfixed fixed	Butyrylthiocholine iodide $6.3 \times 10^{-5} \text{M}$	284 C 51 $10^{-5} \text{M}$
Es ns E	Fresh	$\alpha$ Naphthyl acetate $5.4 \times 10^{-5} \text{M}$	Eserine $10^{-5} \text{M}$
Er ns E	Fixed	$\alpha$ Naphthyl acetate $5.4 \times 10^{-5} \text{M}$	E 600 $10^{-5} \text{M}$

In the present study the results of estimations of esterase activities in the normal ganglion using different substrates and substrate—inhibitor combinations are summarized in Tables II–IV The intensities of the reactions are only studied visually and in the interpretation of the results the following signs are used —  $\pm$  + ++ +++ which can be read as negative very weak or just visible weak moderate and intense

When naphthol ASD acetate was used as substrate the reaction product was coarsely granular throughout and in every type of section so that it was very difficult to determine the exact site of the activity Therefore little attention is paid to the results obtained with this substrate in the following text

No differences between the sexes were observed in the present study

TABLE II *The intensities of the esterase reactions obtained with different substrates and substrate inhibitor combinations in fresh sections*

Substrate and Inhibitor	Ganglion Cell Cytoplasm	Synapses	Satellite Cells	Nerve Fibres
Acetylthiocholine	— to +	++	+++	+++
150 OMPA $10^{-6}M$	— to +	++	—	some ++
284 C 51 $10^{-3}M$	— to +	—	+++	+++
Eserine $10^{-3}M$	—	—	—	—
E 600 $10^{-4}M$	—	—	—	—
Butyrylthiocholine	some ++	—	+++	+++
150 OMPA $10^{-6}M$	—	—	—	—
284 C 51 $10^{-3}M$	some ++	—	+++	+++
Eserine $10^{-3}M$	—	—	—	—
E 600 $10^{-3}M$	—	—	—	—
$\beta$ Naphthyl acetate	+++	+++	+++	+++
150 OMPA $10^{-6}M$	+++	—	—	+
284 C 51 $10^{-3}M$	+++	—	+++	+++
Eserine $10^{-3}M$	+++	—	+	+
E 600 $10^{-3}M$	—	—	—	—
$\alpha$ Naphthyl butyrate	++	—	++	++
150 OMPA $10^{-6}M$	++	—	—	+
284 C 51 $10^{-3}M$	++	—	++	++
Eserine $10^{-3}M$	++	—	±	±
E 600 $10^{-3}M$	—	—	—	—
4 Chloro 5 bromo-indoxyl acetate	+++	—	++	++
150 OMPA $10^{-6}M$	+++	—	—	+ to +++
284 C 51 $10^{-3}M$	+++	—	++	++
Eserine $10^{-3}M$	+	—	—	—
E 600 $10^{-3}M$	—	—	—	—
Naphthol ASD acetate	+	—	+	+
150 OMPA $10^{-6}M$	+	—	+	+
284 C 51 $10^{-3}M$	+	—	+	+
Eserine $10^{-3}M$	+	—	+	+
E 600 $10^{-3}M$	+	—	—	—

## RESULTS

### NORMAL GANGLION

In the presentation of the results the carboxylic esterases are classified according to the principle adopted in this laboratory (Eränkö *et al* 1964 Harkonen 1964 Kokko 1965 Soderholm 1965) The esterases are divided into two main groups ChEs and ns Es ChEs are subdivided into AChE and ns ChE using 150 OMPA and 284 C 51 as specific inhibitors Ns Es are grouped into Es ns E and Er ns E using E 600 as differentiating inhibitor

The most reliable methods for demonstrating the four groups of carboxylic esterases are presented in the following scheme

Enzyme	Type of section	Substrate	Inhibitor
AChE	Fresh postfixd fixed	Acetylthiocholine iodide $6.9 \times 10^{-5} M$	150 OMPA $10^{-5} M$
Ns ChE	Fresh postfixd fixed	Butyrylthiocholine iodide $6.3 \times 10^{-5} M$	284 C 51 $10^{-5} M$
Es ns E	Fresh	$\alpha$ Naphthyl acetate $5.4 \times 10^{-5} M$	Eserine $10^{-5} M$
Er ns E	Fixed	$\alpha$ Naphthyl acetate $5.4 \times 10^{-5} M$	E 600 $10^{-5} M$

In the present study the results of estimations of esterase activities in the normal ganglion using different substrates and substrate—inhibitor combinations are summarized in Tables II-IV The intensities of the reactions are only studied visually and in the interpretation of the results the following signs are used —  $\pm$  + ++ +++ which can be read as negative very weak or just visible weak moderate and intense

When naphthol ASD acetate was used as substrate the reaction product was coarsely granular throughout and in every type of section so that it was very difficult to determine the exact site of the activity Therefore little attention is paid to the results obtained with this substrate in the following text

No differences between the sexes were observed in the present study

TABLE II *The intensities of the esterase reactions obtained with different substrates and substrate inhibitor combinations in fresh sections*

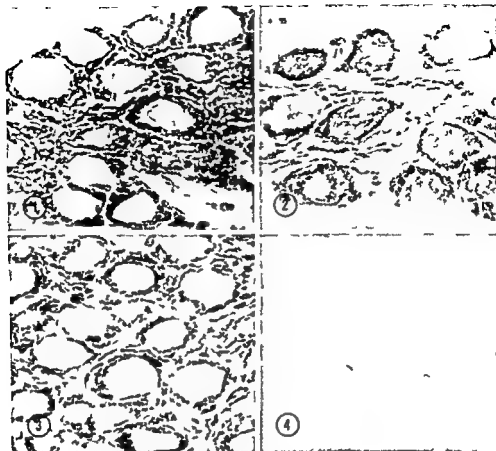
Substrate and Inhibitor	Ganglion Cell Cytoplasm	Synapses	Satellite Cells	Nerve Fibres
Acetylthiocholine	— to +	++	+++	+++
150 OMPA $10^{-4}M$	— to +	++	—	some ++
284 C 51 $10^{-3}M$	— to +	—	+++	+++
Eserine $10^{-3}M$	—	—	—	—
E 600 $10^{-3}M$	—	—	—	—
Butyrylthiocholine	some ++	—	+++	+++
150 OMPA $10^{-4}M$	—	—	—	—
284 C 51 $10^{-3}M$	some ++	—	+++	+++
Eserine $10^{-3}M$	—	—	—	—
E 600 $10^{-3}M$	—	—	—	—
$\alpha$ Naphthyl acetate	+++	+++	+++	+++
150 OMPA $10^{-4}M$	+++	—	—	+
284 C 51 $10^{-3}M$	+++	—	+++	+++
Eserine $10^{-3}M$	+++	—	+	+
E 600 $10^{-3}M$	—	—	—	—
$\alpha$ Naphthyl butyrate	++	—	++	++
150 OMPA $10^{-4}M$	++	—	—	+
284 C 51 $10^{-3}M$	++	—	++	++
Eserine $10^{-3}M$	++	—	±	±
E 600 $10^{-3}M$	—	—	—	—
4 Chloro-5 bromo indoxyl acetate	+++	—	++	++
150 OMPA $10^{-4}M$	+++	—	—	+ to ++
284 C 51 $10^{-3}M$	+++	—	++	++
Eserine $10^{-3}M$	+	—	—	—
E 600 $10^{-3}M$	—	—	—	—
Naphthol AS D acetate	+	—	+	+
150 OMPA $10^{-4}M$	+	—	+	+
284 C 51 $10^{-3}M$	+	—	+	+
Eserine $10^{-3}M$	+	—	+	+
E 600 $10^{-3}M$	+	—	—	—

TABLE III *The intensities of the esterase reactions obtained with different substrates and substrate inhibitor combinations in postfixied sections*

Substrate and Inhibitor	Ganglion Cell Cytoplasm	Synapses	Satellite Cells	Nerve Fibres
Acetylthiocholine	— to ++	+++	+++	+++
iso OMPA 10 <sup>-6</sup> M	— to ++	+++	—	some ++
284 C 51 10 <sup>-5</sup> M	— to +	—	+++	+++
Eserine 10 <sup>-5</sup> M	—	—	—	—
E 600 10 <sup>-5</sup> M	—	—	—	—
Butyrylthiocholine	some ++	—	+++	+++
iso OMPA 10 <sup>-6</sup> M	—	—	—	—
284 C 51 10 <sup>-5</sup> M	some ++	—	+++	+++
Eserine 10 <sup>-5</sup> M	—	—	—	—
E 600 10 <sup>-5</sup> M	—	—	—	—
$\alpha$ Naphthyl acetate	++	—	++	++
iso OMPA 10 <sup>-6</sup> M	++	—	—	—
284 C 51 10 <sup>-5</sup> M	++	—	++	++
Eserine 10 <sup>-5</sup> M	+	—	±	±
E 600 10 <sup>-5</sup> M	—	—	—	—
$\alpha$ Naphthyl butyrate	++	—	++	++
iso OMPA 10 <sup>-6</sup> M	++	—	+	some ++
284 C 51 10 <sup>-5</sup> M	++	—	++	++
Eserine 10 <sup>-5</sup> M	++	—	±	±
E 600 10 <sup>-5</sup> M	—	—	—	—
4 Chloro-5 bromo-indoxyl acetate	+	—	++	++
iso OMPA 10 <sup>-6</sup> M	+	—	—	some ++
284 C 51 10 <sup>-5</sup> M	+	—	++	++
Eserine 10 <sup>-5</sup> M	+	—	—	+
E 600 10 <sup>-5</sup> M	—	—	—	—
Naphthol AS-D acetate	+	—	+	+
iso OMPA 10 <sup>-6</sup> M	+	—	+	+
284 C 51 10 <sup>-5</sup> M	+	—	+	+
Eserine 10 <sup>-5</sup> M	+	—	+	+
E 600 10 <sup>-5</sup> M	+	—	+	+

TABLE IV *The intensities of the esterase reactions obtained with different substrates and substrate inhibitor combinations in fixed sections*

Substrate and Inhibitor	Ganglion Cell Cytoplasm	Synapses	Satellite Cells	Nerve Fibres
Acetylthiocholine	++ to +++	+++	+++	+++
150 OMPA $10^{-6}M$	++ to +++	+++	—	some ++
284 C 51 $10^{-5}M$	— to ++	—	+++	+++
Eserine $10^{-3}M$	—	—	—	—
E 600 $10^{-5}M$	—	—	—	—
Butyrylthiocholine	some ++	—	++	+++
150 OMPA $10^{-6}M$	—	—	—	—
284 C 51 $10^{-5}M$	some ++	—	+++	+++
Eserine $10^{-3}M$	—	—	—	—
E 600 $10^{-5}M$	—	—	—	—
■ Naphthyl acetate	++	—	++	+++
150 OMPA $10^{-6}M$	++	—	—	some +
284 C 51 $10^{-5}M$	++	—	+++	+++
Eserine $10^{-3}M$	++	—	±	±
E 600 $10^{-5}M$	+	—	—	—
α Naphthyl butyrate	++	—	++	++
150 OMPA $10^{-6}M$	++	—	++	+
284 C 51 $10^{-5}M$	++	—	++	++
Eserine $10^{-3}M$	++	—	+	+
E 600 $10^{-5}M$	+	—	—	—
4 Chloro 5 bromo indovyl acetate	++	—	++	++
150 OMPA $10^{-6}M$	++	—	—	some +
284 C 51 $10^{-5}M$	++	—	++	++
Eserine $10^{-3}M$	++	—	±	±
E 600 $10^{-5}M$	+	—	—	—
Naphthol ASD acetate	++	—	+	+
150 OMPA $10^{-6}M$	++	—	+	+
284 C 51 $10^{-5}M$	++	—	+	+
Eserine $10^{-3}M$	++	—	+	+
E 600 $10^{-5}M$	++	—	+	+



Figs 1—4 Esterase reactions obtained with acetylthiocholine. Incubation time 4 hours  
Postfixed sections  $\times 420$

Fig 1 No inhibitor  
Fig 3 284 C 51 10 M

Fig 2 Iso OMPA 10<sup>-3</sup> M (AChE)  
Fig 4 Eserine 10<sup>-3</sup> M

### *Acetylcholinesterase*

*Effect of Inhibitors* In the demonstration of AChE activity in the ciliary ganglion of the rat *iso* OMPA had to be used to inhibit *ns* ChE the activity of which was very intense in the nerve fibres, satellite cells and glial cells as can be seen in Fig. 1. The concentration of *iso* OMPA generally used was 10<sup>-3</sup> M. If the concentration was 10<sup>-2</sup> M or higher *iso* OMPA inactivated a considerable part of the AChE activity in the ganglion cell cytoplasm and in the synapses. On the other hand, if the concentration of *iso* OMPA was

lower than  $10^{-5}M$  the glial tissue exhibited rather strong activity  $10^{-5}M$  284 C 51 totally inhibited the AChE activity in the synapses and positive nerve fibres and in the majority of the ganglion cells (Fig 3)  $10^{-5}M$  eserine and  $10^{-5}M$  E 600 totally inhibited the reaction obtained with acetylthiocholine (Fig 4)

*Other Substrates* Butyrylthiocholine was not hydrolysed by AChE since  $10^{-5}M$  284 C 51 had no effect on the reactivity towards this substrate (Figs 5 and 7) and  $10^{-6}M$  iso OMPA completely inhibited it (Fig 6) In fresh sections  $\alpha$  naphthyl acetate gave a reaction which was partly suppressed by  $10^{-5}M$  284 C 51 (Table II) The inhibition involved the ganglion cell cytoplasm the synaptic zone, and some nerve fibres (Figs 9 and 11) Since no activity was seen in the synaptic area when  $10^{-6}M$  iso OMPA was used as inhibitor (Table II Fig 10) the activity of the synaptic zone revealed by  $\alpha$  naphthyl acetate must have been due to ns ChE in the satellite cells In postfixes and fixed section  $\alpha$  naphthyl acetate was not hydrolysed by AChE since  $10^{-5}M$  284 C 51 had no effect on the reaction (Tables III and IV)

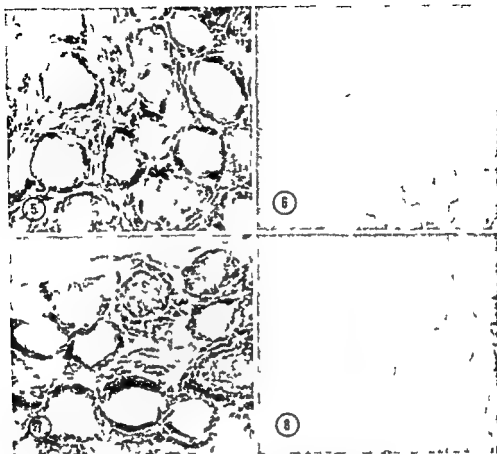
*Distribution* As observed with acetylthiocholine and  $10^{-6}M$  iso OMPA the most intense activity was present in the synapses which appeared like a ring around the neurones (Tables II III and IV Figs 2 21 and 33) These synaptic zones were not always continuous all the way round the cell body but less intensely stained areas were observed at the cell periphery between the synaptic clumps An almost equally strong staining as in the synapses was seen in the cytoplasm of some ganglion cells but there were only a few of these in each ganglion The cytoplasm of other neurones showed a moderate activity in postfixes and fixed sections but only weak to moderate activity in fresh sections The staining was evenly distributed throughout the whole cytoplasm and the reaction product was finely granular In some neurones the nuclear membrane exhibited a more intense activity than the cytoplasm The satellite cells and glial cells were inactive as well as the nuclei and nucleoli but some nerve fibres were intensely reactive In the interstitium between the ganglion cell bodies some strongly positive clumps were observed These were presumed to be synapses of ganglion cells the nucleus and most of the cytoplasm of which were outside the section

A positive reaction was observed in the synapses after incubation for 30 minutes after longer incubation other parts of the ganglion became positive When the incubation time was six hours the distribution of the activity did not change although the intensity of the reaction increased In fresh sections the activity was weaker and the reaction product more diffuse than in postfixes and fixed sections (Tables II III and IV)

### *Non specific Cholinesterase*

*Effect of Inhibitors* When butyrylthiocholine was used as substrate and  $10^{-5}M$  284 C 51 as inhibitor the result was similar to that obtained without an inhibitor, establishing that only ns ChE hydrolysed butyrylthiocholine





Figs 5-8 Esterase reactions obtained with butyrylthiocholine. Incubation time 3 hours  
Postfixed sections  $\times 420$

Fig 5 No inhibitor

Fig 7 284 C 51  $10^{-5}$ M (ns ChE)

Fig 6 Iso-OMPA  $10^{-6}$ M

Fig 8 E 600  $10^{-5}$ M

(Figs 5 7 27 and 39)  $10^{-6}$ M iso OMPA  $10^{-5}$ M eserine and  $10^{-5}$ M E 600 inhibited all the activity obtained with this substrate (Figs 6 and 8)

**Other Substrates** The result with acetylthiocholine as substrate and  $10^{-5}$ M 284 C 51 as inhibitor was similar to that obtained with butyrylthiocholine alone (Tables II III and IV Figs 3 and 5) This reaction was totally inactivated by  $10^{-6}$ M iso OMPA  $\alpha$  Naphthyl acetate  $\alpha$  naphthyl butyrate and 4-chloro 5 bromoindoxyl acetate were also hydrolysed by ns ChE but the e reactions were not totally inhibited by  $10^{-6}$ M iso OMPA (Tables II III and IV Figs 10 and 16) Neither did  $10^{-5}$ M 284 C 51 or  $10^{-5}$ M eserine totally abolish the activity in the cytoplasm of the ganglion cells (Figs 9 11 15 17 and 20)

**Distribution** As shown with butyrylthiocholine the distribution of ns ChE activity was similar in all types of sections although in postfixes and fixed sections the picture was sharper than in fresh sections (Tables II III and IV Figs 5 7 27 and 39) Only a few of the ganglion cells (15 cells per ganglion) were stained moderately to intensely, the other neurones were totally devoid of activity. On the other hand the satellite cells Schwann cells and most of the nerve fibres exhibited intense activity. The reaction product was finely granular. The nuclei and nucleoli of all these types of cells were inactive.

The satellite cells appeared as an intensely stained zone surrounding the ganglion cell bodies like the synaptic ring. Therefore it was difficult to distinguish whether the activity of this zone was due to that of the synapses or of the satellite cells or of both of these. To solve this problem sections stained separately for AChE or ns ChE were counterstained with haematoxylin. From the original sections it was observed that there are not at any rate large amounts of ns ChE capable of hydrolysing butyrylthiocholine present in the synapses. The sections counterstained with haematoxylin established this to be true: the ns ChE activity around the ganglion cell bodies was confined to the satellite cells.

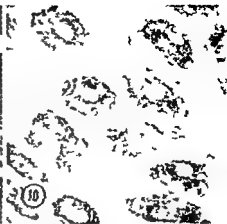
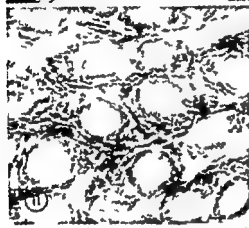
#### *E 600 sensitive Non specific Esterase*

**Effect of Inhibitors** E 600 in a concentration of  $10^{-3}$ M totally inhibited the activity obtained with  $\alpha$ -naphthyl acetate in fresh and postfixes sections (Tables II III and IV Fig 13). In fixed sections some activity due to E r ns E remained.

**Other Substrates** When  $\alpha$ -naphthyl butyrate and 4-chloro 5-bromoindoxyl acetate were used as substrates and  $10^{-3}$ M eserine as inhibitor the distribution of the activity was similar to that observed with  $\alpha$ -naphthyl acetate in fresh postfixes and fixed sections although the intensity of the reaction was a little weaker and the reaction product more coarsely granular than with  $\alpha$ -naphthyl acetate (Tables II III and IV Fig 18).  $10^{-3}$ M E 600 totally inhibited the reaction obtained with these other two substrates in fresh and postfixes sections while in fixed sections the result was closely similar to that obtained with  $\alpha$ -naphthyl acetate and E 600 (Figs 14 and 19).

**Distribution** The enzyme was mainly restricted to the cytoplasm of ganglion cells, the majority of these showing intense activity but some neurones were moderately stained (Figs 12 and 45). The reaction product which was evenly distributed throughout the whole cytoplasm was partly diffuse and partly finely granular and extended some way into the cell processes so that the multipolarity of the ganglion cells could be seen. Weak activity was visible in the cytoplasm of the satellite cells and in some nerve fibres between the ganglion cell bodies. The latter interstitial activity may be partly due to that of the Schwann cells. The nuclei and nucleoli were not reactive.

In the postfixes and fixed sections the distribution of activity was similar to that in the fresh sections but the intensity was a little weaker (Tables



13

14

Figs 9—14 Enzyme reactions obtained with *n*-naphthyl acetate and Blue RR. Incubation time 15 min. Figs 9—13 Fresh sections. Fig 14 Fixed section x420.

Fig 9 No inhibitor

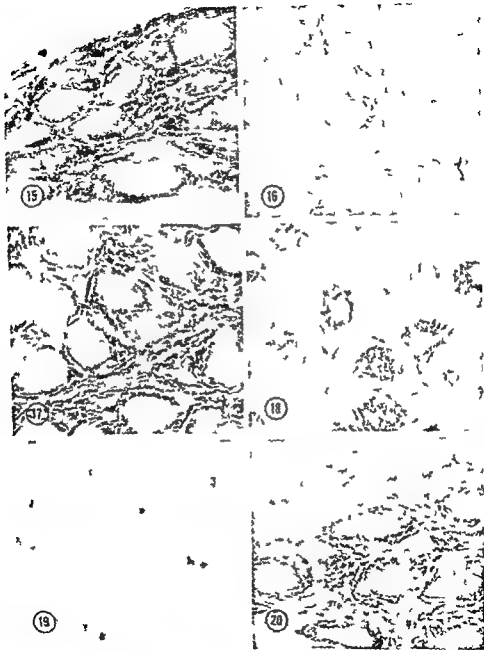
Fig 11 284  $\times 10^{-5}$  M

Fig 13 E 600  $10^{-5}$  M

Fig 10 Iso-OMPA  $10^{-5}$  M

Fig 12 Escrine  $10^{-5}$  M (Es ns F)

Fig 14 E 600  $10^{-5}$  M (Et ns E)



Figs 15—19 Esterase reactions obtained with  $\alpha$ -naphthyl butyrate and Blue RR. Incubation time 25 min Figs 15—18 Postfixed sections Fig 19 Fixed section  $\times 420$   
 Fig 20 Esterase reaction obtained with 4-chloro-5-bromomethyl acetate Incubation time 75 min Postfixed section  $\times 420$

Fig 15 No inhibitor

Fig 16 Iso-OMPA 10 M

III and IV) than in the fresh sections. In addition to this in fixed sections in the interstitium between the ganglion cell bodies, many small and very intensely stained spots were visible (macrophages fibrocytes capillaries?) which had not been affected by any inhibitor (Figs 14 and 19)

#### *E 600 resistant Non specific Esterase*

This enzyme was selectively demonstrated in fixed sections using  $\alpha$  naphthyl acetate as substrate and  $10^{-5}$ M E 600 as inhibitor

*Other Substrates* In fixed sections,  $\alpha$  naphthyl butyrate and 4 chloro 5 bromoindoxyl acetate as substrates, together with  $10^{-5}$ M E 600 as inhibitor gave almost the same picture as that obtained with  $\alpha$  naphthyl acetate except that the intensity of activity was weaker with the two former than with the latter (Fig 19). In fresh and postfixied sections no reaction at all was obtained (Tables II and III)

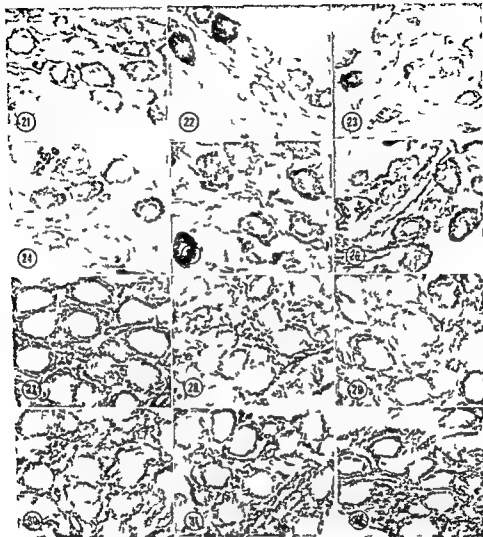
*Distribution* All the ganglion cells showed some activity which was mainly localized in coarse granules but a weak background activity was also seen. The granules were mostly situated perinuclearly as a broad zone but in some neurones the reaction product was concentrated in one end of the ganglion cell (Fig 14). The satellite cells, the glial cells and the nerve fibres as well as all nuclei and nucleoli were totally devoid of activity. In the interstitium between the ganglion cell bodies several intensely stained small clumps were seen. These were homogeneously and heavily stained so that it was impossible to discern the fine structure or determine the nature of these spots

### PREGANGLIONIC DENERVATION

*Acetylcholinesterase* The number of AChE positive synapses around the ganglion cells decreased after the operation. This was first seen on the 3rd day after nerve section and the loss reached a maximum by the 5th day (Figs 21 and 22). Thereafter the number of reactive synapses remained subnormal for about a fortnight (Figs 23 and 24) subsequently gradually returning to normal so that after 60 days the synaptic zones were as densely stained as in the control sections (Figs 25 and 26). The partial weakening of the reaction in the synaptic zone may be due to the degeneration of the preganglionic part of the synapse

The cytoplasmic AChE activity remained substantially unchanged. However this activity seemed to increase during the regeneration period (Figs 25 and 26). The number of active fibres did not change as a result of decentralization

*Non specific Cholinesterase* The ns ChE activity began to decrease 3 days after section of the preganglionic nerve trunk and maximum loss was reached in 5 days (Figs 27, 28, 29 and 30). The formerly strongly positive nerve fibres had lost the greater part of their activity although this decrease



The effect of preganglionic denervation on AChE and ns ChE activities. Incubation time 4 hours for AChE and 3 hours for ns ChE. Postfixed sections  $\times 200$

Fig 21 AChE Control ganglion

Fig 24 AChE 30 days after decentralization

Fig 27 Ns ChE Control ganglion

Fig 30 Ns ChE 15 days after decentralization

Fig 22 AChE 5 days after decentralization

Fig 25 AChE 60 days after decentralization

Fig 28 Ns ChE 5 days after decentralization

Fig 31 Ns ChE 30 days after decentralization

Fig 23 AChE 10 days after decentralization

Fig 26 AChE 90 days after decentralization

Fig 29 Ns ChE 10 days after decentralization

Fig 32 Ns ChE 90 days after decentralization



The effect of postganglionic denervation on AChE and ns ChE activities Incubation time 4 hours for AChE and 3 hours for ns ChE Postfixed sections  $\times 250$

Fig 33 AChE Control ganglion

Fig 36 AChE 15 days after axotomy

Fig 39 Ns ChE Control ganglion

Fig 42 Ns ChE 10 days after axotomy

Fig 34 AChE 1 day after axotomy

Fig 37 AChE 30 days after axotomy

Fig 40 Ns ChE 3 days after axotomy

Fig 43 Ns ChE 30 days after axotomy

Fig 35 AChE 3 days after axotomy

Fig 38 AChE 60 days after axotomy

Fig 41 Ns ChE 5 days after axotomy

Fig 44 Ns ChE 90 days after axotomy

was not so great as that caused by axotomy. The weakening of the fibre activity was due to degeneration of the preganglionic fibres. The activity was partly restored by the 30th day (Fig. 31) and totally normalized by the 60th day after nerve section (Fig. 32). However, in some ganglia the number of active fibres was less than in normal ganglia.

*E 600 sensitive and resistant Non specific Esterases* No distinct changes were found in the activity of either of these enzymes in the ciliary ganglion of the rat after preganglionic denervation.

## POSTGANGLIONIC DENERVATION

*Acetylcholinesterase* The first changes in the activity of this enzyme were already observable on the first postoperative day. The distribution of the AChE reaction in the axotomized ganglion was less sharp than in the control and the cytoplasmic activity of its neurones was clearly weakened (Figs. 33 and 34). After three days most of the ganglion cells were totally negative except for a few neurones whose activity was hardly affected (Fig. 35). The synaptic reactivity had also diminished within less than a day after the operation and AChE positive synapses were less numerous. On the 3rd postoperative day only a few reactive synapses were visible and no AChE positive nerve fibres were seen. The staining remained faint during the following weeks (Fig. 36) but by the 30th day after axotomy activity was somewhat restored (Fig. 37). Two months after the operation the AChE activity in the ganglion cells was at least as strong as in the control ganglion (Fig. 38). By this time synapses and nerve fibres containing AChE had also reappeared.

During the axon reaction the ganglion cell bodies became oval and the nuclei moved to the periphery of the neurone. These phenomena had also normalized 60 days after the operation.

*Non specific Cholinesterase* After 3 days the ns ChE positive nerve fibres had lost their activity (Figs. 39 and 40) although some fibres probably preganglionic were still as strongly positive as in the control section. No active ganglion cells were seen either. The staining remained faint during the following period (Figs. 41 and 42) and gradually began to increase having returned somewhat by the 30th day (Fig. 43) and the activity was totally normalized 60 days after the operation (Fig. 44). The satellite and Schwann cells also lost some of their activity during the axon reaction.





The effect of postganglionic denervation on E-s ns E activity Incubation time 3.5 min  
Fresh sections  $\times 250$

Fig 45 E-s ns E Control ganglion

Fig 48 E-s ns E 5 days after axotomy

Fig 46 E-s ns E 1 day after axotomy

Fig 49 E-s ns E 30 days after axotomy

Fig 47 E-s ns E 3 days after axotomy

Fig 50 E-s ns E 60 days after axotomy

**E 600 sensitive Non specific Esterase** Three days after the operation the activity of this enzyme had definitely decreased in the ganglion cell cytoplasm (Figs 45 46 and 47) The maximum effect was obtained by the 5th day at which time the activity was very weak (Fig 48) After 30 days the reaction was only a little weaker than in the control ganglia (Fig 49) and 60 days after axotomy normalization was complete (Fig 50) By this time the morphological changes observed had also become completely normal

**E 600 resistant Non specific Esterase** The changes were similar to those observed in the case of E-s ns E During the axon reaction the active cytoplasmic granules were less numerous than in the control section and the interstitial heavily stained spots were also greatly diminished in number Both these reappeared during the regenerative period By the 60th day the activity both in the ganglion cells and in the interstitial tissue was totally normalized

### SYMPATHECTOMY

No major changes in the activities of the esterases were detected in the ciliary ganglion of the rat after this type of operation However a loss of some reactive fibres or cells may well have escaped notice since most of the tissue was unaffected by this

## DISCUSSION

## ACETYLCHOLINESTERASE

The staining intensity for AChE was found to vary considerably between the individual neurones in the ciliary ganglion of the rat in the present study. This finding is at variance with those of the previous investigations on other species (Koelle 1951, 1955; Fukuda and Koelle 1959; Koelle and Koelle 1959; McIsaac and Koelle 1959; Cauna *et al* 1961; Taxi 1961; Fredricsson and Sjöqvist 1962; Okinaka *et al* 1963) in which all the ciliary ganglion cells were found to exhibit intense AChE activity even after a relatively short incubation time. However in the present study only a few of the ciliary ganglion cells of the rat possessed a high activity while the majority of the neurones showed a weak to moderate reaction even after prolonged incubation time, a process which only increases the intensity of the reaction but does not affect the distribution of the activity (Holmstedt and Sjöqvist 1959). The possibility that formalin fixation to which AChE is sensitive (Holmstedt and Sjöqvist 1961) was the cause can be excluded since a similar variation between the individual neurones could be seen in fresh sections as well. The different results may partly be due to differences in technique but the most probable cause is that the ciliary ganglion cells of the rat are different from those of the other mammals studied.

In the ciliary ganglion of the rat two types of neurones were found, one exhibiting intense AChE activity and the other weak to moderate activity. A similar differentiation in the AChE activity of individual neurones can be seen in some parasympathetic ganglia (Koelle and Koelle 1959; Cauna *et al* 1961) in sympathetic ganglia (Koelle 1951, 1955; Holmstedt 1957; Giacobini 1960; Taxi 1961; Sjöqvist 1962; Holmstedt *et al* 1963; Harkonen 1964) and in sensory ganglia and other sensory neurones (Koelle 1951, 1955; Giacobini 1960; Esila 1963; Kokko 1965) while the motor neurones usually exhibit a uniformly high AChE activity (Nachmansohn and Hoff 1944; Schwarzscher 1958; Giacobini 1960; Snell 1961; Söderholm 1965).

It has been suggested that the neurones with a high AChE activity are purely cholinergic and those with a low activity non-cholinergic, the latter possessing some transmitter other than ACh (Koelle 1955; Giacobini 1960) although ACh is presumed to have some function in adrenergic transmission too (Burn and Rand 1960, 1965). This is supported by the investigation of Birks and MacIntosh (1961). The adrenergic and sensory neurones usually possess a low or variable AChE activity (Koelle 1955). The nature of those ciliary ganglion cells of the rat that exhibited a low AChE activity has not been determined in the present study although several pharmacological studies suggest that some kind of adrenergic synaptic transmission takes place in the ciliary ganglion of the cat and the dog (Turner-Suden *et al* 1951, 1952; Perry and Talesnik 1953). This view is supported by the present study (see Chapter IV).

Electrophoretically AChE is not homogeneous (Bernsohn *et al* 1962 Eranko *et al* 1964 Harkonen 1964 Kokko 1965 Soderholm 1965) and it is possible that its several forms which may have different functional characteristics are responsible for the variations observed in the staining intensity of the neurones. This might also partly explain the differences in AChE activity in the rat ciliary ganglion cells.

The synaptic terminals which were numerous and formed a continuous zone around the perikarya of some ganglion cells exhibited a higher activity than the neuronal cytoplasm. These results are in keeping with previous studies of the ciliary ganglion cells of mammals and birds as well as of reptiles. This activity is distributed in both pre- and postsynaptic structures in various ganglia including the ciliary (Koelle 1951, 1955; Szentágothai *et al* 1955; Szentágothai 1957; Brown 1958; Koelle and Koelle 1959; Taxi 1961; Fredricsson and Sjoqvist 1962; Harkonen 1964). Biochemical determinations have verified this result (Giacobini 1960). The high AChE activity seen in the synapses suggests a cholinergic transmission in the ciliary ganglion of the rat although the demonstration of AChE alone cannot be taken as absolute proof of the presence of a cholinergic type of transmission (Giacobini 1960; Koelle 1963; Shute and Lewis 1966; Eranko 1967).

The AChE positive nerve fibres were mainly postganglionic axons as demonstrated by the results of the denervation procedures. This finding is in good agreement with those of previous investigations (Koelle 1951; Gerebtzoff 1959; Taxi 1961). The capillaries in the ciliary ganglion of the rat did not show any AChE activity although active capillaries have been seen in the central nervous system (Crook 1963).

Decentralization had no effect on the cytoplasmic AChE activity, as was also seen in previous investigations concerning the ciliary ganglion (Szentágothai *et al* 1955; Koelle and Koelle 1959; Taxi 1961) and other ganglia (Brown 1958; Snell 1958; Holmstedt *et al* 1963; Harkonen 1964). However, the synaptic reactivity diminished somewhat after section of the oculomotor nerve. This may be due to degeneration of the preganglionic synaptic structures upon loss of their enzyme activity. This explanation fits well with the results of previous studies (Szentágothai *et al* 1955; Koelle and Koelle 1959; Taxi 1961). In sympathetic ganglia, in addition to the synaptic changes, disappearance of the preganglionic nerve fibres (Brown 1958; Snell 1958; Koelle and Koelle 1959; Harkonen 1964) and a marked decrease in the AChE concentration of the whole ganglion (v Brucke 1937; Dhar 1958; Harkonen 1964) were observed. The number of reactive nerve fibres did not change in the present study after decentralization which establishes that preganglionic fibres are not AChE positive in the ciliary ganglion of the rat. However, the neurones of the Edinger Westphal nucleus from which the preganglionic nerves of the ciliary ganglion arise exhibited intense AChE activity (Koelle 1954, 1955; Gerebtzoff 1959).

Axotomy brought about many drastic changes in the enzyme activity of the ciliary ganglion. In three days most of the AChE had disappeared so that only a few synapses and neurones still exhibited activity. These ganglion

cells had possibly escaped axotomy. High activity was seen in the proximal axon stump in some sections demonstrating the axonal flow of AChE as reported by other workers (Sawyer 1946 Harkonen 1964 Eranko and Harkonen 1965). Almost complete disappearance of the cytoplasmic axonal and postganglionic synaptic AChE activity has been described as taking place in other ganglia and motor neurones after axotomy (Sawyer and Hollinshead 1945 Dhar 1958 Schwarzacher 1958 Harkonen 1964 Gromadzki and Koelle 1965 Soderholm 1965) followed by functional changes while the production of ACh keeps within normal limits (Brown *et al* 1952 Brown and Pascoe 1954).

According to McLennan (1954) axotomy in the sympathetic ganglion of the cat had no effect on the AChE content of the preganglionic fibres and synaptic structures. In the present study it was therefore surprising to see that most of the preganglionic synaptic terminals had lost their AChE activity after axotomy. Similar changes have also been observed in some previous investigations (Harkonen 1964 Illis 1964 Soderholm 1965).

This change may be explained in several ways. (1) During the operation the preganglionic nerve fibres may have been damaged by stretching or by deficiency of blood supply so that these fibres degenerated and lost their AChE activity (Taxi 1961). (2) Axotomy may really influence the AChE activity of the preganglionic synaptic structures (Sawyer and Hollinshead 1945 Brown 1958). The preganglionic neurone after it observes that the postganglionic neurone is unable to conduct impulses to the effector organ may stop producing AChE. (3) The highly active apparently synaptic zone may in fact not contain any synapses at all but the reactivity may be due to activity in the endoplasmic reticulum concentrated in the periphery of the ganglion cell cytoplasm. However the results of preganglionic denervation do not support this suggestion. (4) The preganglionic neurones may have had some transmitter other than ACh so that AChE activity was not needed in the synapses either.

These suggestions are highly speculative and the problem is still unsolved. Electron microscopic investigations in combination with histochemical methods are necessary for its solution since many ultrastructural changes in the neuronal cytoplasm and synapses have been observed after nerve division (De Robertis 1956 Harkonen 1964 Hunt and Nelson 1965 Lewis and Shute 1965 a).

The changes in enzyme reactivity observed after nerve section were reversible for during the regenerative period activity was normalized. However the length of time between the minimal (or maximal) enzyme activity and its subsequent complete return to normal varies. This is probably due to distance of the denervation sites from the cell bodies (Kawai 1963 Brodal to be published).

In the present study ns ChE was found to be mainly localized in the interstitial tissue which consists of many tissue components satellite cells Schwann cells, pre and postganglionic axons dendrites and capillaries all of which apparently exhibited ns ChE activity although it was difficult to determine the exact site of the esterase activity These results agree well with findings concerning the ciliary ganglia of other mammals (Koelle 1951 1955 Giacobini 1959 1960 Cauna *et al* 1961 Taxi 1961) and also mammalian sympathetic and sensory ganglia (Koelle 1951 1955 Giacobini 1956 1959 Holmstedt 1957 Cauna *et al* 1961 Taxi 1961 Harkonen 1964 Kokko 1965) Some ciliary ganglion cells of the rat exhibited a strong ns ChE activity which has not been reported in any nerve cells of this ganglion in the other mammals studied (Koelle 1951 1955 Giacobini 1959 1960 Cauna *et al* 1961, Taxi 1961) A similar difference between the ganglionic neuronal ns ChE activity of the rat and that of other mammals has been encountered in the sympathetic and sensory ganglia (Koelle 1951 1955 Giacobini 1959 Cauna *et al* 1961 Taxi 1961, Harkonen 1964 Kokko 1965) Ns ChE activity is also present in the central nervous system where it is mainly localized in the glial cells myelinated nerve fibres and capillaries (Hard and Peterson 1949 Ord and Thompson 1952 Cavanagh *et al* 1954 Koelle 1954 1955 a Foldes *et al* 1962 Teoharov 1962) In addition to the nervous system all other tissues including the blood also contain ns ChE (Alles and Hayes 1940 Richter and Croft 1942 Aldridge 1954 Easson and Stedman 1957)

Since ns ChE is so widely distributed in various tissues it must play some role although this is not known Many hypotheses have been put forward Clitherow *et al* (1963) suggested that in the liver it might hydrolyse toxic byproducts of lipid metabolism In brain tissue ns ChE might be involved in  $\gamma$ aminobutyrylcholine metabolism (Holmstedt and Sjoqvist 1960) while in the synaptic structures it might eliminate any stray AChE (Koelle 1962) In the walls of the capillaries ns ChE might participate in the regulation of permeability (Koelle 1955 a) The high ns ChE activity observed in the satellite cells confirms the metabolic or other exchanges taking place between the neurone and its glia (Scharf 1958 Hiden 1960)

As a result of both pre and postganglionic denervation the interstitial ns ChE activity decreased remarkably in a few days and more strikingly after axotomy than after decentralization These results correspond well with those observed in the sympathetic ganglion after denervation (Harkonen 1964) The myelinated nerves as a rule contain ns ChE (Coupland and Holmes 1957) which is mainly localized in the myelin sheath (Cavanagh *et al* 1954 Tewari and Bourne 1960) Therefore the decreased activity observed in the present study after decentralization is readily understandable since this operation brings about the degeneration of myelin sheaths in the distal part of sectioned nerve fibres such as the sympathetic ganglion (Sawyer and Hollinshead 1945) The remaining activity was probably due

to that of postganglionic nerve fibres and satellite and Schwann cells which had not been affected by preganglionic denervation. In degenerating peripheral nerves on the contrary *ns ChE* rose above the normal value (Cavanagh *et al* 1954).

Postganglionic denervation on the other hand should not cause any myelin degeneration in the ganglion. Therefore the great decrease of *ns ChE* activity observed after axotomy must mainly be due to diminished reactivity in the glial cells which might partly result from some non specific factors such as ischaemia caused by the operation (Harkonen 1964). However axotomy may also affect the enzymic activity of the preganglionic structures (Sawyer and Hollinshead 1945). The satellite cells were affected much less than the Schwann cells while the normally positive ganglion cells had totally lost their activity after section of the short ciliary nerves.

### NON SPECIFIC ESTERASES

*Es ns E* exhibited an intense activity in practically all the ciliary ganglion cells of the rat and the variation between individual neurones was less than in the sympathetic (Savay *et al* 1953 Harkonen 1964) and spinal (Thomas 1963 Kokko 1965) ganglia. The *Er ns E* activity was less intense than that of *Es ns E* and it was mainly localized in cytoplasmic granules as has also been shown to be the case in other ganglia (Harkonen 1964 Fisher and Sutherland 1965 a Kokko 1965) and spinal cord neurones (Soderholm 1965). There was also another difference between *Es ns E* and *Er ns E* namely that the latter could only be demonstrated in fixed sections while the former was most active in fresh sections and almost negative in fixed sections. Even postfixation did not immobilize *Er ns E*. Thus the desmo-enzyme nature of *Es ns E* and the lyo-enzyme nature of *Er ns E* were also confirmed in the present study. Further several histochemical and starch gel electrophoretic investigations concerning the nervous tissue have shown this view to be correct (Eranko *et al* 1962 1964 Harkonen 1964 Kokko 1965 Soderholm 1965).

*Es ns E* seems to be bound to the endoplasmic reticulum and is poorly soluble. This enzyme however is very easily inactivated by formalin (Hannibal and Nachlas 1959) so that it is hardly demonstrable at all in fixed material. *Er ns E* is mainly associated with granules (Harkonen 1964 Fisher and Sutherland 1965 Kokko 1965) an observation which has been supported by biochemical and electron microscopic studies (Aldridge and Johnson 1959 Torack and Barnett 1962).

*Ns Es* are widely distributed in living tissues (Nachlas and Seligman 1949 Barnett 1952 Gomori 1953 1955 Mendel *et al* 1953 Markert and Hunter 1959) and they behave in a great variety of ways in relation to substrates or other reagents (Barnett and Seligman 1951 Bergmann *et al* 1957 Burstone 1957 Pearson and Defendi 1957 Underhay 1957 Bergmann and Rimoin 1958). Therefore the concept *ns E* consists of a great variety of

enzymes or enzyme groups the functions of which are not exactly known. Some authors have suggested that they are proteolytic enzymes in nervous tissue (Pearse 1956, Pepler and Pearse 1957, Hess and Pearse 1958) and also in other tissues (Hopsu and Glenner 1963, 1964) while others believe that they hydrolyse lipids (Myers and Mendel 1953, Novikoff 1961). It has also been thought that ns Es may affect the growth and development of lower organisms but that in the course of evolution the latter significance of ns Es has been lost in higher animals (Myers and Mendel 1953).

Decentralization was found to have no effect on ns E activities in the ciliary ganglion of the rat while axotomy caused a marked, but in general, reversible decrease of both E s ns E and E r ns E activities. The behaviour of the latter enzyme differs from that described by Harkonen (1964) and Fisher and Sutherland (1965 a). Many of the E r ns E positive granules had lost their enzyme activity after axotomy as observed by Soderholm (1965) and in the present study.

In size, shape and cytoplasmic distribution these granules, some at least of which give a positive acid phosphatase reaction (Koenig 1962, 1964, Kawai 1963, Sharma 1964), resemble lipofuscin granules (Gedigk and Bontke 1956), lysosomes (De Duve *et al.* 1955, Wachstein and Meisel 1960, Novikoff and Essner 1962, De Duve 1963, Novikoff 1963, Goldfischer *et al.* 1964, Fisher and Sutherland 1965 a) or dense bodies (Torack and Barnett 1962). Some granules are also autofluorescent (Kokko 1965). After axotomy however the E r ns E positive granules behave in a dissimilar way to the acid phosphatase positive ones since the latter increase after the operation (see Chapter II). Therefore all these granules cannot be identical.

The decreased activity of ns Es after postganglionic denervation can be explained in the following way: after axotomy anabolic processes are greatly intensified in the cell body (Brattgård *et al.* 1957) and synthesizing enzymes, are needed while catabolic enzymes like hydrolases (e.g. esterases) are not. Therefore the former increase and the latter decrease after axotomy (Soderholm 1965).

## II PHOSPHATASES

### EARLIER INVESTIGATIONS

#### NORMAL NERVOUS TISSUE

##### *Acid Phosphatase*

The author is unaware of any publication dealing with phosphatase activities in the ciliary ganglion but other nervous tissues have been studied. A weak acid phosphatase (AcPase) reaction has been shown to occur in the stellate ganglion cells of the rat (Smith and Luttrell 1947 Smith 1948) while in the sympathetic ganglia of the guinea pig and man an intense staining has been observed (Walter 1953 Anderson and Son, 1962). In the spinal ganglion of the rat the small cells exhibited a stronger activity than the larger ones (Tewari and Bourne 1962 c Kokko 1963) in the trigeminal and nodose ganglia of the rat (Tewari and Bourne 1963 1964) and the monkey (Fisher and Sutherland 1963a) the variation in AcPase activity between individual cells was not so great. In the motor neurones of the spinal cord of the monkey (Bodian and Mellors 1943) the guinea pig (LaVelle *et al* 1954) and the rat (Soderholm 1963) a high activity has been reported.

The distribution of AcPase is mainly cytoplasmic while the nuclear staining sometimes seen has been suggested to be an artifact (Eranko 1951 Barron and Sklar 1961 Deane 1963). Tewari and Bourne (1962) have reported AcPase in the synapses of the rat cerebellum and suggested that this enzyme might possess some function in synaptic transmission as well.

Examined under a light microscope the neuronal AcPase seems to be localized in granules especially in fixed sections (Samorajski and Fitz 1961 Fisher 1963 Kokko 1963) and electron microscopic studies have confirmed these results (Novikoff 1963 Goldfischer *et al* 1964 Sobel and Avrin 1965). The AcPase-containing granules or dense bodies may be lysosomes some near the Golgi apparatus and others in the endoplasmic reticulum (Novikoff and Essner 1962 Koenig 1963 Novikoff 1963 Osinchak 1963 Sobel and Avrin 1965). It has also been thought that AcPase is manufactured in ribosomes and then transported via the Golgi apparatus to the lysosomes (Goldfischer *et al* 1964 Sobel and Avrin 1965). Some authors have reported that lipofuscin granules contain AcPase (Gedigk and Bontke 1956 Koenig 1964) while Barka (1962) suggested that the AcPase-positive structures may have been derived from pinocytotic processes.



### *Alkaline Phosphatase*

In a study on the sympathetic ganglion of the guinea pig and man Walter (1955) reported a strong alkaline phosphatase (AlPase) activity in the capillaries the nerve fibres, the connective tissue and elsewhere in the perineuronal tissue while the ganglion cell cytoplasm was devoid of activity with the exception of the nuclear membrane and some fibrils within the nuclei. In the semilunar ganglion of the cow the ganglion cell cytoplasm showed a reaction in fresh sections which varied from strong to negative (Scharf and Rowe 1957). The interstitial tissue was also intensely reactive. In the spinal and trigeminal ganglia of the rat a diffuse perineuronal activity has been reported in addition to the strong reaction in the capillaries in fresh and fixed sections (Tewari and Bourne 1962c 1963 1964).

In the rat brain and spinal cord capillaries have usually been reported to be the only active sites (Becker *et al* 1960 Fishman and Havash 1962 Soderholm 1965) but Tewari and Bourne (1962 1963c) and Nandy and Bourne (1963) have found AlPase activity in the synaptic regions as well. In the rabbit spinal cord nerve cell processes and glial nuclei stained positively (Simorajski and Fitz 1961).

### *Adenosine Triphosphatase*

In the spinal and trigeminal ganglia of the rat Tewari and Bourne (1962c 1963 1963a) have reported a positive reaction for adenosine triphosphatase (ATPase) in the nerve cells in addition to intense activity in the nerve fibres and capillaries. The activity varied considerably between individual ganglion cells. Some activity was also seen in the nuclei and nucleoli (Sandler and Bourne 1962).

In the spinal cord of the rat and the guinea pig ATPase is said to be mainly confined to the neurones (the cell membrane the mitochondria the endoplasmic membranes the nuclei and the nucleoli) and capillaries (Wawrzyniak 1963 Nandy and Bourne 1964). However Soderholm (1965) did not observe any intracytoplasmic reaction in the anterior horn cells of the rat spinal cord. In the rat brain ATPase is distributed in much the same way as in the spinal cord (Becker *et al* 1960 McClurkin 1964 Torack 1965). Nardoo (1962) has emphasized the high nuclear activity. No synaptical activity has been reported in the rat cerebellum (Tewari and Bourne 1963b).

With the aid of the electron microscope ATPase has been localized in the glial neuronal contact areas at the surface of neurones and in the distal synaptic regions of the axon as well as in the glial processes adjacent to the walls of the capillaries. Within the neurones the most prominent sites of activity of this enzyme were the Golgi apparatus and the proximal parts of the axon (Torack and Barnett 1963). In myelinated nerve fibres ATPase activity was confined exclusively to the axoplasm (Torack 1965). In other tissues such as the liver intestine musculature etc ATPase was concentrated in the mitochondria (Lazarus and Barden 1962 Goldfischer *et al* 1964 Schulze and Wollenberger 1965). As determined biochemically the glial cells exhibited a higher ATPase activity than the nerve cells in Deiters nucleus in the rabbit the neurones however exhibited a high activity just inside the cell membrane (Cummins and Hyden 1962 Hyden 1962). Germain and Proulx (1965) have isolated synaptic vesicles containing ATPase from the rat brain.

## EFFECT OF DENERVATION

### *Preganglionic Denervation*

There is no report available concerning the effect of preganglionic nerve division on the activities of the phosphatases. Such a study is of obvious interest in view of the reports claiming synaptic localization of phosphatases (Texari and Bourne 1962 1963) e Nandy and Bourne 1963).

### *Postganglionic Denervation*

**Acid Phosphatase:** There are numerous histochemical investigations in which an increase in AcPase activity has been reported in the motor neurones of the spinal cord of several species after section of the sciatic nerve (Bodian and Mellors 1943 Lassek and Bueker 1947 Smith 1948 Bueker *et al* 1949 Barron and Sklar 1961 Barron and Tuncbay 1962 Iwama 1963 Soderholm 1965). Samorajski and Fitz (1961) are the only authors who did not observe any such histochemically demonstrable change in the AcPase reaction of the rabbit spinal cord. An increase of activity after axotomy has also been observed in the facial nuclei of the rabbit (Coimbra and Tavares 1964) in the hypoglossal nuclei of the cat (Barron and Sklar 1961) and in the dorsal vagal ganglia of the monkey (Fisher and Sutherland 1963a). Similar changes have been demonstrated in the stellate and nodose ganglia of the cat after postganglionic denervation (Smith and Luttrell 1947 Smith 1948).

Biochemical determinations showed increased activity in the grey matter of the axotomized spinal cord of the rabbit to about three times the normal value while the activity in the white matter did not change significantly (Samorajski and Fitz 1961). On the other hand Fieschi and Soriana (1959) found no change in the AcPase activity in the spinal cord of the guinea pig after bilateral section of the sciatic nerve.

The increased AcPase activity after axotomy was reversible in all the cases studied.

**Alkaline Phosphatase:** A lowered ALPase activity has been established biochemically in the spinal cord of the guinea pig and of the rabbit after sciatic nerve section (Fieschi and Soriana 1959 Samorajski and Fitz 1961) while no histochemical changes were seen in the spinal cord of the rabbit (Samorajski and Fitz 1961) or the rat (Soderholm 1965) after axotomy.

**Adenosine Triphosphatase:** A few days after unilateral sciatic nerve section the anterior horn cells of the rat spinal cord exhibited a strongly increased reaction at the cell membrane the activity returning to normal in about two months (Soderholm 1965). Biochemically a reduction in ATPase concentration has been detected in the spinal cord of the guinea pig after bilateral sciatic nerve division (Fieschi and Soriana 1959).

## METHODS

Fresh, frozen and fixed sections were used. In the case of ATPase postfixation was performed in formal-calcium for 5-10 minutes at -4 to 0°C (Wachstein *et al* 1962). In the routine work only postfixed sections were used.

Gomori's (1952) methods for acid and alkaline phosphatases were employed a mixture of equal parts of sodium  $\alpha$  and  $\beta$ -glycerophosphate being used as substrate. The incubation time was 60 minutes for AcPase and 30 minutes for AlPase at 37°C.

ATPase was demonstrated with the method described by Wachstein and Meisel (1957). The incubation time was from 6 to 10 minutes at 37°C.

Controls were made by incubating the sections in a solution from which the substrate was omitted. Controls for AcPase were also made by incubating the sections in the usual solution to which  $10^{-3}$ M sodium fluoride was added (Newman *et al.* 1950). All these controls were always negative.

## RESULTS

### NORMAL GANGLION

#### *Acid Phosphatase*

This enzyme was best demonstrated in postfixed sections because with fixed sections a non-specific precipitate which could not be rinsed off was found all over the section. The activity was mainly distributed in the ganglion cell cytoplasm and was partly diffuse and partly granular. The diffuse activity was weak and evenly distributed throughout the cytoplasm. The active coarse granules on the other hand were mainly accumulated perinuclearly sometimes in clumps although an even distribution was also seen in some neurones. The nucleoli were sometimes positive but in the majority of ganglion cells the nuclei were totally devoid of activity. A few satellite cells also exhibited some activity which was weaker however than in the neurones. In the interstitium some activity could be seen in a few sections it was probably located in the Schwann cells (Figs 51 and 53).

In fresh sections the activity was mostly diffuse so that only blurred granules could be seen. The interstitial structures exhibited a higher activity than that found in postfixed sections.

#### *Alkaline Phosphatase*

The capillaries were intensely stained. Some black granular deposits were also seen at the periphery of some of the ganglion cells (Fig. 57). When the incubation time was prolonged to two hours a continuous black ring was observed round the ganglion cell bodies but the reaction was so diffuse that it was impossible to localize the activity exactly. The nuclei and nucleoli were negative.

The results were similar in fresh postfixed and fixed sections.

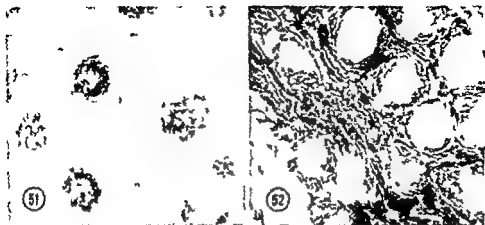


Fig 51 Acid phosphatase Incubation time 60 min Postfixed section  $\times 420$

Fig 52 Adenosine triphosphatase Incubation time 6 min Postfixed section  $\times 420$

### *Adenosine Triphosphatase*

A weak finely granular reaction was observed in the peripheral parts of the cytoplasm of some of the ganglion cells and this reaction was somewhat intensified by prolonged incubation. Surrounding the ganglion cell bodies a strongly positive zone was seen. It was very difficult however to localize this activity more exactly; it may partly be due to that of the cell membranes partly to that of cell processes or synaptic structures or satellite cells. Nerve fibres and glial cells exhibited an intense activity as did also the capillaries which were the components most intensely stained. The nuclei and nucleoli were totally negative (Figs 52 and 59).

A similar distribution pattern of enzyme activity was observed in fresh and postfixed sections but in the former the reaction product was more diffuse than in the latter.

### PREGANGLIONIC DENERVATION

No distinct changes were observed in the activities of any of the phosphatase studied after decentralization in the ciliary ganglion of the rat although it seemed as if the ATPase activity decreased slightly after the operation. However this change was not significant.

## POSTGANGLIONIC DENERVATION

*Acid Phosphatase* On the 5th postoperative day AcPase showed a somewhat increased activity in the neurones (Figs 53 and 54). The maximum intensity of the reaction was reached in 10 days (Fig 55) after which the activity remained for a fortnight at this high level. The active cytoplasmic granules seemed to be more numerous and to stain more intensely than those of the control ganglion. In some neurones these particles had mostly accumulated in the axon hillock and the corresponding axon exhibited a strong reaction for some distance. The interstitial tissue showed no change in its activity after axotomy.

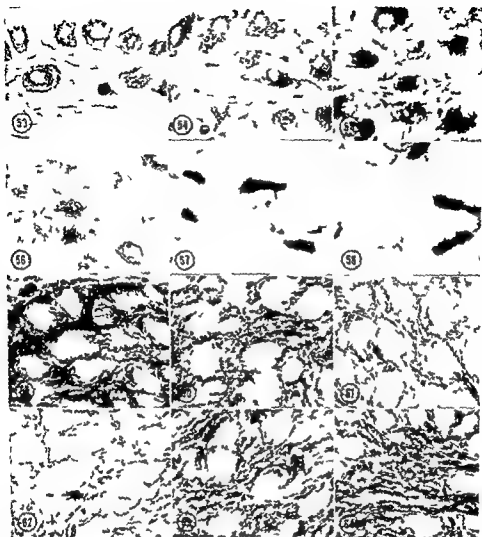
One month after neurotomy the reaction was still slightly stronger than in the control but by the 60th day the intensity of activity and the distribution and number of the positive granules were the same as in the contralateral ganglion (Fig 56).

*Alkaline Phosphatase* No significant changes were observed in the activity of this enzyme in the ciliary ganglion of the rat after axotomy (Figs 57 and 58).

*Adenosine Triphosphatase* Five days after postganglionic denervation the ATPase activity in the ganglion had slightly decreased (Figs 59 and 60) and the minimum level was reached by 10 to 15 days. At this time the interstitial activity was weak with the exception of the capillaries which still gave an intense reaction. The persisting activity seemed to be due to that of the ganglion cell membrane and of the satellite and glial cells in addition to the capillaries. Thus only the nerve fibres had lost most of their ATPase (Figs 61 and 62). By the 30th postoperative day the interstitial activity was somewhat restored (Fig 63) and by the 60th day the reaction had totally normalized (Fig 64).

## SYMPATHECTOMY

No clearly discernible changes were observed in the phosphatase activities in the ciliary ganglion of the rat after extirpation of the ipsilateral superior cervical ganglion.



The effect of postganglionic denervation on phosphatase activities. Incubation time 60 min for AcPase, 30 min for AlPase and 6 min for ATPase. Postfixed sections  $\times 250$ .

Fig 53 AcPase Control  
ganglion  
Fig 56 AcPase 60 days  
after axotomy  
Fig 59 ATPase Control  
ganglion  
Fig 62 ATPase 15 days  
after axotomy

Fig 54 AcPase 5 days  
after axotomy  
Fig 57 AlPase Control  
ganglion  
Fig 60 ATPase 5 days  
after axotomy  
Fig 63 ATPase 30 days  
after axotomy

Fig 55 AcPase 10 days  
after axotomy  
Fig 58 AlPase 15 days  
after axotomy  
Fig 61 ATPase 10 days  
after axotomy  
Fig 64 ATPase 60 days  
after axotomy

## DISCUSSION

## ACID PHOSPHATASE

There was a marked difference in the cytoplasmic distribution of AcPase in fresh and fixed sections. In the former the reaction product was so diffuse that granules could hardly be seen. In postfixes sections however although these did sometimes also exhibit evidence of slight diffusion and in fixed sections the activity was mainly concentrated in granules. Thus we are again faced with the question of whether AcPase is partly lyo and partly desmo-enzyme as was theoretically proposed by Lison (1948) and actually shown by Eranko (1952), Nachlas *et al* (1956), Hannibal and Nachlas (1959) and Kokko (1965). It is impossible however to solve this problem by histochemical methods alone. In the living organism AcPase may be localized exclusively within granules but the many technical procedures before and during staining may rupture these particles and release the enzyme into the soluble cytoplasm (Bitensky 1963, De Duve 1963, Tappel *et al* 1963). Otherwise basically the results observed accord well with those of previous studies on other ganglia.

Decentralization had no effect on the AcPase activity in the ciliary ganglion of the rat since the main activity was cytoplasmic so that degeneration of the preganglionic fibres did not change the general impression gained.

The changes observed in AcPase activity in the axotomized ciliary ganglia are in keeping with the results of previous investigations concerning other neuronal tissue.

The axon reaction causes an increase in the protein synthesis of the cell body (Brattgard *et al* 1957) for which phosphate derivatives are required; these are most easily supplied by increasing the phosphatase activity (LaVelle *et al* 1954). A high AcPase activity has in fact been demonstrated in the secretory cells (Eranko 1951, Novikoff 1961, Sobel and Avrin 1965) suggesting that this enzyme is involved in synthetic processes.

On the other hand Fisher and Sutherland (1965a) have observed that an initial increase is followed by a decrease in AcPase activity in the neurones of the nodose ganglion and the dorsal vagal nuclei of the monkey. According to these workers AcPase has a catabolic effect since the initial increase in the activity of the enzyme coincides well with chromatolysis whereas a decreased activity was observed during the recovery period at which time chromosynthesis is taking place. In the present study no such lowered activity was seen.

## ALKALINE PHOSPHATASE

A high AlPase activity was observed exclusively in the capillaries of the rat ciliary ganglion as in other nervous tissue (Becker *et al* 1960, Fishman and Hayashi 1962, Bannister and Romanul 1963, Soderholm 1965).

Neither pre nor postganglionic denervation had any effect on the AlPase

activity in the ciliary ganglion of the rat. This fits in well with previous histochemical studies dealing with the spinal cord (Samorajski and Fitz 1961 Soderholm 1965). The existence of normal activity in the capillaries of the denervated ganglia established that the blood supply had not been seriously disturbed if at all by the operations.

#### ADENOSINE TRIPHOSPHATASE

ATPase in the ciliary ganglion cells was mainly localized in the cell membrane as has previously been seen in other neurones (Becker *et al* 1960 Cummins and Hyden 1962 Soderholm 1965) although some positive intracytoplasmic particles which may have been mitochondria or endoplasmic structures containing ATPase (Goldfischer *et al* 1964 Nandy and Bourne 1964) were also seen. The cytoplasm of most ganglion cells was negative which may have been due to inhibition of the enzyme since ATPase is extremely sensitive (Myers and Slater 1957 Wachstein *et al* 1962).

The most important function of ATPase is to release energy by hydrolysing adenosine triphosphate (ATP) and so to furnish the energy required for the ion pump and thus for active transport as a rule across cell membranes (Cummins and Hyden 1962 Tewari and Bourne 1962 c 1963 b). Therefore a high ATPase activity is necessary at the surface of the capillary glial and neuronal cell membranes. The propagation of nerve impulse involves the movements of ions across the conductive membrane and the energy required to restore the ionic imbalance of the quiescent membrane can be obtained by hydrolysing ATP. Thus axonal dendritic and synaptic ATPase activity becomes explicable (Hyden 1962).

After avotomy the interstitial ATPase activity weakened considerably in the ciliary ganglion of the rat and the decrease can be explained as a consequence of axonal flow of the enzyme since it has been reported that the axoplasm contains ATPase (Totack 1965). However this explanation is highly speculative. Increased activity as seen in the motor neurones of the spinal cord after sciatic neurotomy (Soderholm 1965) was not encountered in the present study.



### III OXIDATIVE ENZYMES

#### EARLIER INVESTIGATIONS

#### NORMAL NERVOUS TISSUE

##### *Dehydrogenases*

**NADH and NADPH Tetrazolium Reductases** As regards NADH (reduced nicotinamide adenine dinucleotide or reduced diphosphopyridine dinucleotide DPNH) and NADPH (reduced nicotinamide adenine dinucleotide phosphate or reduced triphosphopyridine dinucleotide TPNH) tetrazolium reductases otherwise known as DPN- and TPN-diaphorase respectively a wide variation in the intensity and intracellular distribution of activity has been shown to exist between individual neurones in the sympathetic and sensory ganglia of mammals. A positive reaction is demonstrable in all the ganglion cells especially in their perikarya. The activity is usually stronger in them than in the neuropil in which it is mainly localized in the satellite Schwann and glial cells while the axoplasm exhibits only weak activity. As a rule DPN-diaphorase gives a more intense reaction than TPN-diaphorase especially in the neuronal cytoplasm. The nuclei and nucleoli are devoid of activity (Potanos *et al* 1959 Romanul and Cohen 1960 Smorajski 1960 Thomas and Pearse 1961 Harkonen 1964).

A very similar distribution pattern is found in the spinal cord (Smorajski 1960 Kumamoto and Bourne 1963 Soderholm 1965) and brain tissue (Becker *et al* 1960 Thomas and Pearse 1961 Friede and Fleming 1962 Rubinstein *et al* 1962) of mammals.

In the pre and postsynaptic structures in the spinal cord of the rat Nandy and Bourne (1964b) have demonstrated DPN-diaphorase positive granules while Soderholm (1965) did not observe any such reaction.

**NAD and NADP linked Tetrazolium Reductases** NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate) linked tetrazolium reductases generally called dehydrogenases are enzymes concerned in carbohydrate protein and fat metabolism.

In the ciliary ganglia of birds and reptiles succinate dehydrogenase has been described as occurring as intensely stained granules in the neuronal cytoplasm while in the submandibular ganglion of the rat the Schwann cells possess the highest activity. No synaptic activity for the enzyme was seen in these ganglia (Szentgothai 1957). However in the synaptic areas of the spinal cord of the rat succinate dehydrogenase positive granules have been seen (Nandy and Bourne 1964b) but Soderholm (1965) did not observe any.

In ganglion cells and in other neurones glutamate alcohol succinate and  $\alpha$  glycerophosphate dehydrogenases show weak activity while satellite cells and neuroglia exhibit strong reactions in tests for succinate and  $\alpha$  glycerophosphate dehydrogenases (Hess and Pearse 1961 Kumamoto and Bourne 1963 Harkonen 1964 Ferguson 1965 Soderholm 1965). The reactivity of the latter enzyme can be intensified if menadione (2 methyl 1 4 naphthoquinone) is used as intermediate electron acceptor (Hess and Pearse 1961).

As to other dehydrogenases such as isocitrate lactate and malate tetrazolium reductases ganglionic tissue gives a positive reaction which is stronger in the nerve cell bodies than in the neuropil. Such a picture has been described in the sympathetic ganglion of the rat (Harkonen 1964). In the sensory ganglia of mammals the small ganglion cells have usually been found to possess higher dehydrogenase activity than the larger ones; neuroglia exhibits only weak activity (Scharf and Rowe 1957 Klein 1960 Romanul and Cohen 1960 Thomas and Pearse 1961 Tewari and Bourne 1963).

In the spinal cord of the rat (Nandy and Bourne 1964a Soderholm 1965) and in the brain tissue of mammals (Padykula 1952 Robins and Smith 1953 Mustakallio 1954 Shimizu and Morikawa 1957 Shimizu *et al.* 1957 Friede 1960 Friede and Fleming 1962 Tewari and Bourne 1962) the grey matter as a rule exhibits strong dehydrogenase activity compared with that of the white matter which however also possesses some activity (Wolfgram and Rose 1959).

Biochemical studies of the rabbit brain have demonstrated a higher succinate dehydrogenase content in the white matter than in the grey (Kawakita 1956). With a microgasometric technique corresponding results have been obtained concerning the distribution of this enzyme in the spinal ganglion of the rabbit (Hyden *et al.* 1958). These results thus support the histochemical findings. However glutamate dehydrogenase exhibits a higher activity than malate dehydrogenase in the neurones of Deters nucleus in the rabbit while the histochemical picture is the opposite (Hamberger 1961).

## Oxidases

**Cytochrome Oxidase (CytO).** This enzyme formerly known as indophenol oxidase has been described as giving a positive and mainly granular reaction in the perikarya and synaptic regions in the ciliary ganglia of birds and reptiles and in the submandibular ganglion of the rat (Szentagothai 1957). Cytochrome positive granules have also been encountered in the synapses of the spinal cord of the rat (Nandy and Bourne 1964b).

In all the sympathetic ganglion cells of the rat a positive reaction has been observed; in capsular cells and other interstitial tissue exhibited weaker activity (Harkonen 1964). In the trigeminal ganglion of the newborn rat an intense reaction was observed in all the ganglion cells (Burstone 1961) while in the spinal ganglion of the rat activity varied between individual neurones from weak to intense (Tewari and Bourne 1962a).

In the brain tissue the grey matter possesses strong activity while the white matter almost negative (Meeph and Pope 1950 Shimizu *et al.* 1957 Burstone 1959 Hannibal *et al.* 1960). The activity is especially intense in the neuronal cell bodies dendritic processes and axonal terminals although in the cerebellum of the rat Tewari and Bourne (1962) have demonstrated strong activity in the white matter too.

**Monoamine Oxidase (MAO)** In the ciliary ganglion of the cat the perikarya were all stained with varying intensity but granularity was weak. In addition in the granular reaction a homogeneous purplish background staining was observed in the cytoplasm of the nerve cells. Nerve fibres and their terminations also exhibited similar background plus granular staining while other interstitial structures were negative. In the rabbit the ciliary ganglion cells were only faintly stained (Koelle and Valk 1954).

A strong granular and weak diffuse MAO activity has been described in the sympathetic and sensory ganglion cells of the cat (Koelle and Valk 1954), the guinea pig (Glenner *et al* 1957) and the rat (Harkonen 1964, Klingman and Klingman 1966). This activity was less intense in the rabbit than in the cat tissue (Koelle and Valk 1954). The ganglion cells of Auerbach's and Meissner's plexuses also exhibited a high activity (Eder 1957, Wohlrab 1961). In the central nervous system of rodents MAO has a mainly neuronal distribution (Arioka and Tanimukai 1957, Shimizu *et al* 1959) although Tewari and Bourne (1963d) described a stronger reaction in the white matter than in the grey in the rat cerebellum.

### *Intracellular Distribution of Oxidative Enzymes*

From histochemical studies it can be seen that both dehydrogenases and oxidases are partly localized in cytoplasmic granules which have been assumed to be mitochondria (Hughes 1956). Friede and Pax (1961) established this for succinate dehydrogenase and CytO while Walker and Seligman (1963) added to this group  $\beta$ -hydroxybutyrate lactate malate and isocitrate dehydrogenases. Many cytochemical investigations have established that enzymes of the tricarboxylic acid cycle in addition to those of the cytochrome system and to  $\beta$ -hydroxybutyrate dehydrogenase are associated with mitochondria (Siekevitz and Watson 1956, Pearse *et al* 1958, Scarpelli and Pearse 1958). Becker *et al* (1960) suggested that DPN-diaphorase is located in mitochondria and ergastoplasm whereas TPN-diaphorase is only present in mitochondria. These results are supported by Vesco and Giuditta (1966).

Biochemical studies have also demonstrated that both CytO and MAO are located in mitochondria (Brody *et al* 1952, Hogeboom and Schneider 1952, Blaschko *et al* 1957, Weiner 1960).

Electron microscopically NADH-tetrazolium reductase and succinate dehydrogenase are seen to be localized exclusively in mitochondria (Ogawa and Barnett 1965).

## EFFECT OF DENERVATION

### *Preganglionic Denervation*

No change was observed after decentralization in either the dehydrogenase or oxidase activities of the superior cervical ganglion of the rat (Harkonen 1964).

### *Postganglionic Denervation*

**Dehydrogenases** An increase in all the dehydrogenase activities was seen in the sympathetic ganglion cells of the rat after axotomy. The change was as a rule reversible. The activities of dehydrogenases were concentrated in a central region of the axotomized neurone (Harkonen 1964). In the spinal ganglion of the rat increased succinate dehydrogenase activity was seen after sciatic nerve section and at the same time the activity of the enzyme was intensified in the proximal stump of the axon indicating axonal flow of the enzyme (Klein 1960).

In the motor neurones of the rat spinal cord the succinate and NADPH tetrazolium reductase activities diminished after axotomy (Friede 1959) while in the guinea pig Kumamoto and Bourne (1963) observed a slight increase in DPN-diaphorase activity and a decreased succinate dehydrogenase activity in both the spinal cord and ganglion. Soderholm (1965) described an increase in the activities of dehydrogenases in the anterior horn cells of the rat spinal cord after axotomy with the exception of a glycerophosphate menadione and succinate tetrazolium reductase the activities of which did not change. TPN-diaphorase and a few dehydrogenases exhibited increased activity in the neurones of the facial nuclei of the guinea pig (Kreutzberg 1963) and the rabbit (Fisher and Malik 1964) after division of the ipsilateral facial nerve.

Biochemically succinate dehydrogenase activity has been demonstrated to decrease somewhat in the axotomized spinal cord of the monkey (about 12%) (Howe and Flexner 1947) and the guinea pig (about 7%) (Kumamoto and Bourne 1963). In the latter species a greater decrease (about 15%) was observed in the spinal ganglion while lactate dehydrogenase did not undergo any significant change. On the other hand Harkonen (1964) demonstrated an increase in the lactate and glucose-6-phosphate dehydrogenase activities in the sympathetic ganglion of the rat after axotomy.

**Oxidases** Histochemically decreased Cytochrome activity after axotomy has been established in the sympathetic ganglion cells and in the interstitial structures of the rat (Harkonen 1964) and also in the spinal cord of the guinea pig (Kumamoto and Bourne 1963). The number of active granules diminished in the nerve cells. These results coincide well with those of biochemical studies concerning the thalamic nuclei and the spinal cord of the cat and the monkey after decortication and axotomy respectively (Howe and Mellors 1943). However the latter workers did not observe any temporal correlation between chromatolysis and Cytochrome concentration. Therefore they suggested that the decrease in the Cytochrome content was due to degeneration of some neurones rather than to the axon reaction.

After axotomy MAO activity decreased considerably in the superior cervical ganglion of the rat although an initial increase was observed. This enzyme also exhibited axonal flow (Harkonen 1964).

## METHODS

*Dehydrogenases*

**NADH and NADPH Tetra-olium Reductases** The method described by Scarpelli *et al* (1958) was used. NADH and NADPH were used as substrates and nitro blue tetrazolium salt (2,2-di-p-nitrophenyl 5,5-diphenyl 3,3'-[3,3'-dimethoxy-4,4'-biphenylene] distetrazolium chloride) abbreviated NBT was used as final electron acceptor. The incubation time was 30 minutes for DPV-diaphorase and 60 minutes for TPN-diaphorase at 37°C.

**NAD linked Tetra-olium Reductases** The method described by Hess *et al* (1958) was employed. The substrates used were  $\beta$ -hydroxybutyrate, isocitrate, malate, glutamate, lactate,  $\alpha$ -glycerophosphate and ethanol. NBT was used as final electron acceptor. The incubation time was 60 minutes for glutamate and alcohol dehydrogenase and 30 minutes for the other enzymes at 37°C.

**NADP linked Tetra-olium Reductases** Glucose-6-phosphate as substrate and NBT as final electron acceptor were used to demonstrate glucose-6-phosphate dehydrogenase according to the method of Hess *et al* (1958). The incubation time was 45 minutes at 37°C.

**$\alpha$ -Glycerophosphate Menadione Tetra-olium Reductase** Sodium  $\alpha$ -glycerophosphate as substrate, menadione as co-factor and NBT as electron acceptor were used to demonstrate this enzyme according to the method described by Wattenberg and Leong (1960) and Wattenberg (1961). The incubation time was 30 minutes at 37°C.

**Succinate Tetra-olium Reductase** Sodium succinate as substrate and NBT as final electron acceptor were used to demonstrate this enzyme according to the method of Nachlas *et al* (1957). The incubation time was 45 minutes at 37°C. The effect of phenazine methosulphate (PMS) in a concentration of 30  $\mu$ g/ml of the incubation medium was studied as well.

A study was also made of the effect of 5 minutes postfixation in cold acetone on the intracytoplasmic distribution of various dehydrogenases. The sections were well rinsed in distilled water before incubation (Novikoff *et al* 1960).

The controls were made in three ways: (1) The sections were incubated in a medium from which the substrate was omitted and the endogenous substrates were washed away by preincubation in a buffer solution for 5 minutes. (2) Co-enzyme or in the case of succinate dehydrogenase NBT was omitted from the incubation solution. (3) The enzymes were inactivated by heating the sections for 3–5 minutes at 80°C in distilled water before incubation. These controls were always negative.

*Oxidases*

**Cytochrome Oxidase** This enzyme was demonstrated according to Burstone's method (1959, 1960, 1961) as modified by Harkonen (1964). The incubation time was 45–60 minutes at room temperature.

Controls were made by omitting the substrate from the incubation solution or by destroying the enzyme by heating as in the case of dehydrogenases. These controls were totally devoid of activity. When potassium cyanide in a concentration of 10 'M' was added to the incubation medium the activity was also totally inhibited.

**Monamine Oxidase** Both the method described by Koelle and Valk (1954) and that presented by Glenner *et al* (1957) were used to demonstrate this enzyme. The former method as modified by Harkonen (1964) was used routinely because of the superior intensity of the reaction. The incubation time was 11 hours at 37°C.

Controls were made by incubating the sections without substrate or in the presence of 10 'M' Marild (isooctyl) isopropyl hydrazine phosphate) in both the preincubation and incubation media. These controls were totally negative.

## RESULTS

## NORMAL GANGLION

*Dehydrogenases*

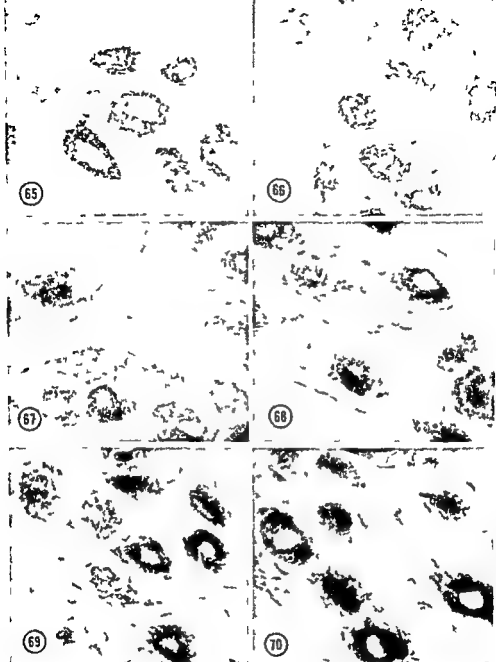
*NADH and NADPH-Tetrazolium Reductases* DPN diaphorase activity was moderate to intense in the ciliary ganglion cells between which some variation in the intensity of staining was observed. All the neurones were positive. Both diffuse and granular activity were seen. The particles were usually evenly distributed throughout the whole cytoplasm but in some nerve cells they had accumulated in the central parts. The diffuse staining in the ganglion cell cytoplasm was even and weak. It was difficult to determine with certainty whether there was really any activity at the sites of the synapses. In the satellite cells a few large granules could be seen, their distribution being mainly perinuclear. However, the reactivity of the capsular cells was much weaker than that of the ganglion cells. In the neuropil only a few positive granules were seen in addition to the very weak interstitial background staining; the particulate activity was probably localized in the Schwann and glial cells. The nuclei and nucleoli were totally devoid of activity (Figs 65 and 77).

TPN diaphorase activity differed somewhat from that of the preceding enzyme. The intensity of the neuronal cytoplasmic reaction was weaker than that of DPN-diaphorase in spite of the longer incubation time. The mean size of the positive granules was larger and their number less than in the case of DPN-diaphorase. The interstitial structures exhibited an equally strong reaction as for DPN-diaphorase. The nuclei and nucleoli were negative (Fig. 66).

*NAD<sup>+</sup> linked Tetrazolium Reductases* Moderate  $\beta$ -hydroxybutyrate dehydrogenase activity was localized mainly in small granules but there was also a weak diffuse cytoplasmic staining. The particles were mostly concentrated in the central regions of the neurones. A slight reaction was also observed in the nuclei and nucleoli. The cytoplasmic activity varied somewhat between individual nerve cells. The other tissue components exhibited both granular and diffuse activity higher than that of DPN diaphorase (Fig. 67).

*Isocitrate, malate and lactate tetrazolium reductases* all exhibited strong activity in all the ciliary ganglion cells. In addition to the weak diffuse cytoplasmic staining, intensely positive particles were observed which in most neurones were distributed around the unstained nuclei. These enzymes showed the highest dehydrogenase activity in the ciliary ganglion cells of the rat. The interstitial tissue components were stained as with DPN diaphorase (Figs 68, 69 and 70).

*Glutamate tetrazolium reductase* exhibited only a weak and mainly granular activity in all the neurones of the ciliary ganglion. Nucleoli in the otherwise unstained nuclei were positive in some neurones. The interstitial tissue including the satellite cells was also weakly stained (Fig. 71). No



Oxidative enzyme activities Fresh sections x 420

Fig 65 NADH tetrazolium reductase  
NBT incubation time 30 min

Fig 67  $\beta$ -Hydroxybutyrate tetrazolium  
reductase NBT incubation time 30 min

Fig 69 Malate tetrazolium reductase  
NBT incubation time 30 min

Fig 66 NADH tetrazolium reductase  
NBT incubation time 60 min

Fig 68 Isocitrate tetrazolium  
NBT inc. 30

Fig 70  
NBT 30



Oxidative enzyme activities Fresh sections  $\times 420$

Fig 71 Glutamate tetrazolium reductase  
NBT incubation time 60 min

Fig 73  $\alpha$ -Chlorophosphate tetrazolium  
reductase NBT incubation time 30 min

Fig 75 Aspartate oxidase Incubation  
time 60 min

Fig 72 Glucose 6-phosphate tetrazolium  
reductase NBT incubation time 45 min

Fig 74 Succinate tetrazolium reductase  
NBT incubation time 45 min

Fig 76 Monoamine oxidase (Koelle  
Valk) Incubation time 2 hours



positive reaction for *alcohol dehydrogenase* could be detected in the cells of the ciliary ganglion. However all over the sections discrete granules were seen which were probably due to the lipid solubility of the tetrazolium salt used. If the incubation time was prolonged for 2—3 hours no further activity was observed with either of these two enzymes.

**NADP<sup>+</sup> linked Tetrazolium Reductase** The only representative of this group in the present study *glucose 6 phosphate dehydrogenase*, exhibited a moderate activity in all the ganglion cells. Most of the activity was diffuse the granules were less numerous and were evenly distributed throughout the cytoplasm. Interstitial structures were stained as in the case of other dehydrogenases. The nuclei were totally inactive (Fig 72).

■ **Glycerophosphate Menadione Tetrazolium Reductase** A moderate reaction was seen in all the ganglion cells. The granules were evenly distributed throughout the cytoplasm. The diffuse activity was weak. The nuclei were devoid of any activity. Around a few neurones a granular precipitate was found which in some sections was very intense. This reaction was probably nonspecific rather than synaptic because these granules were also of a different colour than those in the neuronal cytoplasm. The interstitial tissue showed the weak reaction (Fig 73). If NAD was substituted for menadione a weaker reaction was obtained in the ganglion cells.

**Succinate tetrazolium reductase** was strongly positive in the ganglion cells and mainly granular although a weak diffuse cytoplasmic background staining was also seen. The granules were mainly perinuclearly distributed. The nuclei were totally unstained. Other components of the ganglion exhibited a reaction similar to that of DPN diaphorase (Figs 74 and 89). The addition of PMS to the incubation solution enhanced the reactivity without affecting the quality or distribution of the reaction product.

After postfixation in cold acetone the diffuse cytoplasmic reaction of the dehydrogenases studied was less strong than in the fresh sections and so rendered the picture sharper. The granular activity was unchanged.

## Oxidases

**Cytochrome oxidase** All the neurones were moderately stained and some activity was also seen to extend into the cell processes. The staining of the individual cells varied somewhat in intensity. The distribution of the granules which were numerous was mostly perinuclear. A weak diffuse staining was observed in the cytoplasm of the ganglion cells. The satellite cells and neuropil exhibited a stronger activity than in the case of the dehydrogenases but weaker than that in the nerve cell bodies. Some of the nerve fibres were intensely stained these were probably myelinated. The weak interstitial activity was partly granular and partly diffuse. The nuclei and nucleoli were negative (Figs 75 and 94).

**Monoamine Oxidase** This enzyme almost exclusively exhibited a diffuse and evenly distributed cytoplasmic activity which was equally intense in all the ganglion cells. Only a few active granules were observed in the periphery.

of the neuronal cytoplasm. The satellite cells were negative and the neuropil gave a very weak diffuse reaction. The nuclei and nucleoli were devoid of activity (Fig. 76).

The hydrazone method of Koelle and Valk gave a more intense staining than Glenner's formazan method. However, the distribution and nature of the reaction product were exactly the same with the two methods.

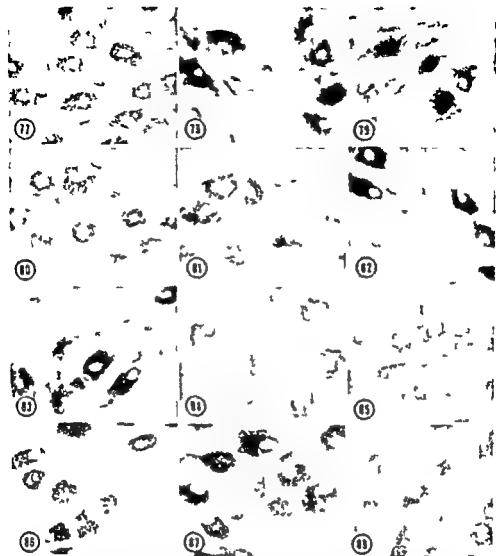
### PREGANGLIONIC DENERVATION

No distinct changes were observed in the intensity or distribution of the activities of any oxidative enzymes studied in the ciliary ganglion of the rat after decentralization.

### POSTGANGLIONIC DENERVATION

*Dehydrogenases*. NADH and NADPH tetrazolium reductases exhibited a pronounced and progressive increase in activity from the first postoperative day onwards. The maximal reaction was reached by the 5th day (Figs. 77 and 78) and a high level persisted for about a fortnight (Fig. 79). On the 30th day after the operation the activity had decreased a little and reached the normal level by the 60th day (Fig. 80). Both diffuse and granular activity were intensified. The positive granules were chiefly concentrated in the central parts of the neuronal cytoplasm while the nuclei adopted an eccentric position. The granules were larger and more intensely stained than in the control ganglion cells. Some neurones gave only a weak reaction; these cells were probably suffering from a deficient blood supply. The activity in the satellite cells and in the neuropil did not change after axotomy. DPN diaphorase activity increased much more than that of TPN diaphorase.

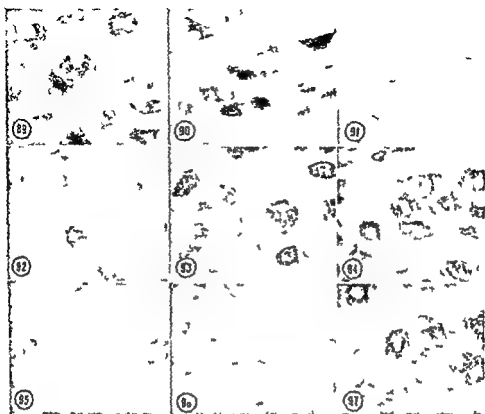
*NAD<sup>+</sup> linked tetrazolium reductases* (only isocitrate and lactate dehydrogenases were studied after axotomy) exhibited increased activity even on the first postoperative day and the maximal activity was reached in 3 to 5 days (Figs. 81, 82, 85 and 86). Thereafter the high level was maintained for about 2 weeks (Figs. 83 and 87). After a month the activity was only a little higher than in the control and was totally normalized within two months after the operation (Figs. 84 and 88). The intracellular changes in the enzyme activity were similar to those of diaphorases. No significant change was found in the satellite cells or in the neuropil.



The effect of postganglionic denervation on oxidative enzyme activities. Fresh sections  
x 250

Figs 77—80 NADH tetrazolium reductase NBT Incubation time 30 min Figs 81—84  
Isocitrate tetrazolium reductase NBT Incubation time 30 min Figs 85—88 Lactate  
tetrazolium reductase NBT Incubation time 30 min

- |                              |                              |                              |
|------------------------------|------------------------------|------------------------------|
| Fig 77 Control ganglion      | Fig 8 5 days after axotomy   | Fig 9 10 days after axotomy  |
| Fig 80 60 days after axotomy | Fig 81 Control ganglion      | Fig 82 5 days after axotomy  |
| Fig 83 15 days after axotomy | Fig 84 90 days after axotomy | Fig 85 Control ganglion      |
| Fig 86 5 days after axotomy  | Fig 87 15 days after axotomy | Fig 88 60 days after axotomy |



The effect of postganglionic denervation on oxidative enzyme activities  
Fresh sections  $\times 250$

Figs 89—93 Succinate tetrazolium reductase NBT Incubation time 30 min Figs 94—97  
Cytochrome oxidase Incubation time 60 min

Fig 89 Control ganglion	Fig 90 3 days after axotomy	Fig 91 10 days after axotomy
Fig 92 30 days after axotomy	Fig 93 60 days after axotomy	Fig 94 Control ganglion
Fig 95 5 days after axotomy	Fig 96 10 days after axotomy	Fig 97 60 days after axotomy

$NADP^+$  linked tetrazolium reductase glucose 6 phosphate dehydrogenase exhibited a change in activity after axotomy similar to that of other dehydrogenases. However the change was not so marked as that of  $NAD^+$  linked dehydrogenases.

■ Glycerophosphate menadione tetrazolium reductase behaved in a similar way to the former enzyme. Both particulate and diffuse staining increased. The granules occupied the centre of the nerve cell beside the nucleus which adopted an eccentric position.

*Succinate dehydrogenase* however behaved differently from all the other dehydrogenases studied. In the majority of ganglion cells a moderate increase in the activity of this enzyme was found during the first five postoperative days while other neurones possessed normal activity (Figs 89 and 90). Thereafter the activity began to decrease in all the neurones reaching a minimum 10 days after axotomy (Fig 91), and then remained at this low level for about 2 weeks. After a month the activity was only a little weaker than in the control ganglion (Fig 92) and in 60 days it had returned to normal (Fig 93). Both particulate and diffuse activity were affected. Only the ganglion cells showed a change in the level of this enzyme while the interstitial tissue components retained their normal activity.

*Oxidases* *Cytochrome oxidase* exhibited a somewhat decreased activity in the ganglion cells from the 3rd postoperative day onwards. The weakest reaction was observed on the 5th day (Figs 94 and 95). Almost as weak an activity was also seen on the following days (Figs 96) but it gradually began to intensify and reached its normal level by the 60th day (Fig 97). The normally strongly positive granules were less numerous during the axon reaction while the diffuse activity was as strong as in the control sections. The reactivity of the interstitial structures did not show any change after axotomy.

*Monoamine oxidase* activity exhibited behaviour similar to that of Cytochrome oxidase after axotomy.

## SYMPATHETOMY

No clearly discernible change was found in the enzyme activities of the dehydrogenases and oxidases studied in the ciliary ganglion after extirpation of the ipsilateral superior cervical ganglion.

## DISCUSSION

## DEHYDROGENASES

The histochemically demonstrable activities of the tetrazolium reductase studied varied considerably in the ciliary ganglion cells of the rat. The relative activities visually estimated were as follows — the most active DPN-diaphorase, isocitrate malate and lactate dehydrogenases, the moderately active TPN diaphorase, succinate  $\beta$ -hydroxybutyrate, glucose 6-phosphate and  $\alpha$ -glycerophosphate menadione tetrazolium reductases, the weakly positive glutamate dehydrogenase, the nonactive alcohol dehydrogenase. Of these enzymes, isocitrate, succinate and malate dehydrogenases take part in Krebs cycle,  $\alpha$ -glycerophosphate, lactate and alcohol dehydrogenases in glycolysis, glucose 6-phosphate dehydrogenase in the pentose phosphate cycle,  $\beta$ -hydroxybutyrate dehydrogenase in the metabolism of fatty acids and glutamate dehydrogenase in amino acid metabolism.

The dehydrogenase activities in the ciliary ganglion of the rat mainly resembled those in the sympathetic and sensory ganglia of mammals (Potanos *et al.* 1959, Klein 1960, Romanul and Cohen 1960, Samorajski 1960, Thomas and Pearse 1961, Tewari and Bourne 1963, Harkonen 1964). However, in the sympathetic ganglion cells of the rat, succinate and  $\alpha$ -glycerophosphate menadione tetrazolium reductases were weakly positive and glutamate dehydrogenase devoid of activity (Harkonen 1964), while in the ciliary ganglion cells these enzymes were as reactive as other dehydrogenases, with the exception of glutamate dehydrogenase which was only weakly positive as observed in the present study. The greatest differences compared with the motor neurones of the rat spinal cord were seen in the distribution of the succinate and  $\alpha$ -glycerophosphate menadione tetrazolium reductases. These enzymes were mostly localized in the neuropil of the spinal cord (Soderholm 1965), while in the ciliary ganglion the staining of the neuronal cytoplasm was far more intense.

The ciliary ganglion cells also showed individual variations in their reactions with different substrates, although this variation was not great. Such a variation in staining intensity may be due to differences in the phase of the metabolic cycle (Tewari and Bourne 1962a, 1962b). In the present study, however, no correlation was found between the intense perinuclear staining and the location of the nucleolus. The difference in staining between the neurones of the same ganglion may also be due to heterogeneity in the enzyme activity of mitochondria (Hess *et al.* 1958, Scarpelli *et al.* 1958). Szentágothai (1957) and Nandy and Bourne (1964b) have demonstrated some oxidative enzyme activity in the synaptic structures in the ciliary ganglion and in the spinal cord. No such reaction could be detected with certainty in the ciliary ganglion of the rat because it was masked by the activity in the satellite cells. However, the synaptic terminals contain mitochondria (De Robertis 1956) so that the activity of oxidative enzymes in synapses is understandable.

The neuronal cytoplasmic reactivity was both particulate and diffuse. This was also clearly seen in the case of succinate dehydrogenase an enzyme which has been considered to be localized exclusively in mitochondria (Scarpelli and Pearse 1958 Rodriguez De Lores Arnaiz and De Robertis 1962 Ogawa and Barnett 1965). The diffuse activity may be due to the disrupted mitochondria but in some cases it may also be due to microsomal activity as in the case of DPN-diaphorase (Vesco and Giuditta 1967).

Some part of the reactivity may also be due to nonspecific staining. The lipid solubility of tetrazolium salts may bring about such an effect and it is thought that all the myelin reactivity may have been due to such an artifact—substantivity of formazans (Pearse and Hess 1961). On the other hand tetrazolium salts may also be reduced to insoluble formazan without any enzymatic activity—nothing dehydrogenase—probably owing to the reaction of the highly reactive sulphhydryl groups in the preparation (Zimmermann and Pearse 1959 Shaw and Koen 1965). These authors stated that alcohol dehydrogenase is largely responsible for nothing dehydrogenase activity. Menadione and phenazine methosulphate are also able to bring about such a reaction (Hashimoto *et al.* 1963).

The glial cells also exhibited some activity although they reacted much less intensely than the neurones. Similar activity has also been demonstrated in the sympathetic ganglion of the rat (Härkönen 1964). A low oxidative metabolism in the neuroglia has been postulated in many biochemical studies (Robin and Smith 1953 Lowry *et al.* 1954 Strominger and Lowry 1955) while Hyden and Pigeon (1960) have demonstrated a high cytochrome and succinate oxidase reactivity in the oligodendroglial cells of Deters' nuclei. The degree of glial activity may have been due to the tetrazolium salts used since it has been shown that the glial tissue reduces other tetrazolium salts better than the neuronal enzymes (Wolffgram and Rose 1959 Rossi and Tsou 1961). The glial activity observed in the present study was not due to nothing dehydrogenase or substantivity of formazans because the boiled sections and the sections incubated without substrate were totally negative. This observation is corroborated by Härkönen (1964). Therefore oxidative metabolic processes are also taking place in the satellite cells and neuroglia which confirms the close metabolic relationship between the neurone and its glia (Hydén 1960).

All the dehydrogenases with the exception of succinate tetrazolium reductase exhibited increased activity in the ciliary ganglion cells after axotomy as long as decentralization had any effect. The intracellular distribution was also shifted to the central parts of the neurones. These results agree well with the earlier investigations concerning the motor neurones and the sympathetic ganglion cells (Kreutzberg 1963 Härkönen 1964 Söderholm 1965). Succinate dehydrogenase behaves in dissimilar ways in different neurones and in different species after axotomy (Howe and Mellors 1945 Kumamoto and Bourne 1963 Härkönen 1964 Söderholm 1965).

The increased reduction of tetrazolium salts after axotomy has been suggested to be due to the increased number of mitochondria (Hartmann

1954 Causey and Hoffman 1955 Hudson *et al* 1961), to the swelling of mitochondria (Hartmann 1954 Harkonen 1964) to the dehydration which causes both these changes (Niemi *et al* 1960) and so on. Apparently axotomy with its consequences increases the permeability of the mitochondrial membrane to the substrate and/or tetrazolium salt the result being the histochemically intensified reaction. The number of formazan deposits is greater in the swollen mitochondria (Niemi *et al* 1960).

The increase in the activities of many dehydrogenases is attributed to the enhanced metabolism and synthetic processes in the axotomized ganglion cells as demonstrated by biochemical studies (Brattgård *et al* 1957).

## OXIDASES

Cytochrome oxidase which is widely distributed in the tissues concerned with aerobic metabolism (Rozin 1955) exhibited high activity in the ciliary ganglion cells of the rat. The neuroglia gave a stronger reaction than with dehydrogenases. Similar results have been observed in the spinal and sympathetic ganglia (Hyden *et al* 1958 Harkonen 1964).

Monoamine oxidase activity was also seen in the ciliary ganglion of the rat although this is believed to be a cholinergic ganglion. However the ciliary ganglion cells of the cat and the rabbit exhibited MAO activity as well (Koelle and Valk 1954). The reaction product was mainly nongranular in the present study although MAO is considered to be a mitochondrial enzyme (Blaschko *et al* 1957 Weiner 1960). Therefore the validity of the reaction was suspected. The control sections however were totally negative thus indicating a true positive reaction and this as supported by the changes observed after axotomy. In adrenergic ganglion cells the MAO activity is also partly diffuse and partly granular (Koelle and Valk 1954 Harkonen 1964).

The presence of MAO in a cholinergic ganglion such as the ciliary does not necessarily mean that it is of significance in the oxidation of the adrenergic transmitter substance but MAO may also be involved in the metabolism of other amines (Koelle and Valk 1954).

While decentralization had no effect on the activities of the oxidases in the ciliary ganglion of the rat axotomy caused a definite decrease in both CytO and MAO activity. Similar results have been observed in the sympathetic ganglion (Harkonen 1964) and in the spinal ganglion (Howe and Mellors 1945). The diminution of enzyme activity may indicate a gradual adaptation of the neurones to the decrease in the total amount of axoplasm as suggested by Howe and Flexner (1947).



The neuronal cytoplasmic reactivity was both particulate and diffuse. This was also clearly seen in the case of succinate dehydrogenase an enzyme which has been considered to be localized exclusively in mitochondria (Scarpelli and Pearse 1958, Rodriguez De Lores Arnaiz and De Robertis 1962, Ojawa and Barnett 1965). The diffuse activity may be due to the disrupted mitochondria but in some cases it may also be due to microsomal activity as in the case of DPN-diaphorase (Vesco and Giuditta 1966).

Some part of the reactivity may also be due to nonspecific staining. The lipid solubility of tetrazolium salts may bring about such an effect and it is thought that all the myelin reactivity may have been due to such an artifact, substantivity of formazans (Pearse and Hess 1961). On the other hand tetrazolium salts may also be reduced to insoluble formazan without any enzymatic activity, 'nothing dehydrogenase' probably owing to the reaction of the highly reactive sulphhydryl groups in the preparation (Zimmermann and Pearse 1959, Shaw and Koen 1965). These authors stated that alcohol dehydrogenase is largely responsible for 'nothing dehydrogenase' activity. Menadione and phenazine methosulphate are also able to bring about such a reaction (Hashimoto *et al* 1963).

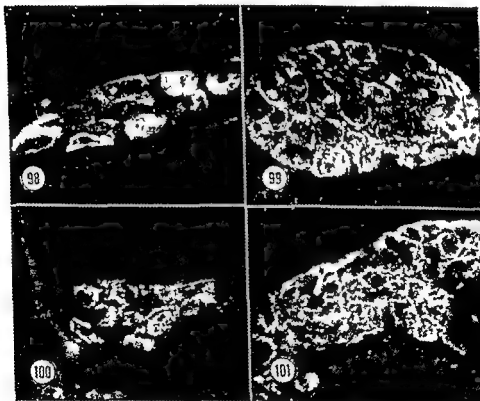
The glial cells also exhibited some activity although they reacted much less intensely than the neurones. Similar activity has also been demonstrated in the sympathetic ganglion of the rat (Härkönen 1964). A low oxidative metabolism in the neuroglia has been postulated in many biochemical studies (Robins and Smith 1953, Lowry *et al* 1954, Strominger and Lowry 1955) while Hydén and Pigon (1960) have demonstrated a high cytochrome and succinic oxidase reactivity in the oligodendroglial cells of Deters nucleus. The degree of glial activity may have been due to the tetrazolium salts used since it has been shown that the glial tissue reduces other tetrazolium salts better than the neuronal enzymes (Wolfgram and Rose 1959, Rosa and Tsou 1961). The glial activity observed in the present study was not due to 'nothing dehydrogenase' or substantivity of formazans because the boiled sections and the sections incubated without substrate were totally negative. This observation is corroborated by Härkönen (1964). Therefore oxidative metabolic processes are also taking place in the satellite cells and neuroglia which confirms the close metabolic relationship between the neurone and its glia (Hydén 1960).

All the dehydrogenases with the exception of succinate tetrazolium reductase exhibited increased activity in the ciliary ganglion cells after axotomy as long as decentralization had any effect. The intracellular distribution was also shifted to the central parts of the neurones. These results agree well with the earlier investigations concerning the motor neurones and the sympathetic ganglion cells (Kreutzberg 1963, Härkönen 1964, Söderholm 1965). Succinate dehydrogenase behaves in dissimilar ways in different neurones and in different species after axotomy (Howe and Mellors 1945, Kumamoto and Bourne 1963, Härkönen 1964, Söderholm 1965).

The increased reduction of tetrazolium salts after axotomy has been suggested to be due to the increased number of mitochondria (Hartmann

## RESULTS

A green fluorescent zone was seen to surround nearly all the ciliary ganglion cell bodies. The fluorescence was of moderate intensity but very short lived. In the ganglion cell cytoplasm some yellow fluorescent granules could be seen and only a few perikarya exhibited the green diffuse fluorescence typical of catecholamines. The granular yellow fluorescence was seen in fresh sections even without treatment with formaldehyde vapour so that it was probably due to prelipofuscin or other autofluorescent granules. The nuclei were always non fluorescent. Some nerve fibres also exhibited the green fluorescence (Figs 98 and 99).



Figs 98—99 Formaldehyde induced fluorescence. Normal ganglion.  
Freeze-dried preparations  $\times 280$

Figs 100—101 Formaldehyde induced fluorescence. Animal treated with nialamide (25 mg/kg intraperitoneally) 8 hours and noradrenaline (15 mg/kg intraperitoneally) 2 hours before death. Freeze-dried preparations  $\times 280$

A number of rats were injected intraperitoneally with nialamide (N-isonicotinoyl-N- $\beta$ -[N-benzyl-carboxyamido] ethyl/hydrazine) 250 mg/kg in a single dose and 5-15 hours later with noradrenaline 1-5 mg/kg in a single dose. The animals were killed 2 hours after the injection of noradrenaline.

As a result of this procedure the formaldehyde induced green fluorescence was much more brilliant and also more permanent. Its localization however did not change but the varicose terminals were more clearly seen (Figs 100 and 101).

The control ganglia, taken from animals whose catecholamine stores had been depleted with reserpine (5 mg/kg daily three doses) did not exhibit any specific fluorescence.

## DISCUSSION

Since the zonal fluorescence was green and totally absent from the control ganglia and since it developed rapidly it seems probable that it was due to noradrenaline (NA) (Falck *et al.* 1962; Falck and Ouman 1965). Some pharmacological data indeed suggest the possible presence of adrenergic synapses in the ciliary ganglia of some mammals (Whitteridge 1937; Tum Suden *et al.* 1951, 1952; Perry and Talesnik 1953; Perry 1957; Martin and Pilar 1963). It has been reported that the neurones of the Edinger Westphal nucleus of the rat contain noradrenaline (Dahlstrom and Fuxe 1965).

The distribution of the fluorescent material around the nerve cell bodies coincided with the synaptic terminals. It is interesting that in the ciliary ganglia of the cat such structures are very few (Hamberger *et al.* 1965) whereas in the rat practically all the ganglion cells were found in the present study to be surrounded by a fluorescent ring of this kind. However it was difficult to determine whether the catecholamines were located in the pre- or postganglionic synaptic structures. Some evidence however, suggests the former site. With the electron microscope 'dense-core' vesicles can be seen in the preganglionic synaptic terminals of the ciliary ganglion of the rat (to be published). These vesicles are presumed to store amines (De Robertis and Pellegrino De Iraldi 1961; Pellegrino De Iraldi *et al.* 1963; Bondareff 1965; Eranko 1967). Furthermore the perikarya of the ciliary ganglion cells did not usually exhibit any specific fluorescence as would be the case if the catecholamines were localized in the postganglionic synaptic structures as they are in the sympathetic ganglia (Eranko and Harkonen 1963, 1965).

From the fact that the preganglionic synaptic terminals also contain AChE which disappears after decentralization (see Chapter I) it can be concluded that there are terminals containing both AChE and NA in the ciliary ganglion of the rat and possibly such terminals are also present in the rat iris in which some nerve fibres contain both AChE and NA (Eranko and Raisänen

1963 Ehinger and Falck 1965 Eranko 1967) The presence and function of MAO in the ciliary ganglion cells thus becomes comprehensible (Kopin 1964)

The significance of the adrenergic synapses in the parasympathetic ganglia is unknown. It has been suggested that adrenaline and NA may inhibit ganglionic transmission and that ACh is the excitatory substance (Hamberger *et al* 1965)

## SUMMARY

The present histochemical investigation deals with the normal activity and distribution of carboxylic esterases phosphatases oxidative enzymes and catecholamines in the ciliary ganglion of the rat and the effect of pre and postganglionic nerve division. The effect of extirpation of the superior cervical ganglion on the activities of hydrolytic and oxidative enzymes was also studied.

**Carboxylic Esterases** (Chapter I) The carboxylic esterases were demonstrated in fresh postfixed and fixed sections using acetylthiocholine iodide butyrylthiocholine iodide  $\alpha$ -naphthyl acetate  $\alpha$ -naphthyl butyrate 4-chloro-5-bromoindoxyl acetate and naphthol ASD acetate as substrates. With the aid of 284 C 51 iso OMPA eserine and E 600 as selective inhibitors the esterases were divided into four groups: acetylcholinesterase (AChE), non-specific cholinesterase (nsChE), E 600 sensitive non-specific esterase (Es nsE) and E 600 resistant non-specific esterase (Er nsE).

AChE was demonstrable in all the ganglion cells the intensity of the reaction varying from weak to strong. The synapses and postganglionic nerve fibres were strongly positive while only a few if any of the preganglionic nerve fibres gave a reaction. Preganglionic denervation abolished the AChE activity of the preganglionic nerve fibres and the preganglionic synaptic terminals. Postganglionic denervation resulted within three days in the total disappearance of AChE from the majority of the ganglion cells including both pre and postganglionic synaptic structures and nerve fibres. The AChE reactivity had to some extent reappeared by the 30th day after the operation and was totally normalized by the 60th day.

The nsChE activity was strong in all the nerve fibres in the satellite and Schwann cells and in the capillaries. Some ganglion cells also exhibited an intense activity. After decentralization the interstitial nsChE activity was much decreased while axotomy caused a still greater decrease in the interstitial nsChE activity. In the latter case the perikarya of the active neurones lost their activity. The activities were normalized in 60 days.

Es nsE activity was seen in all the ganglion cells. In fresh sections the activity was intense indicating the desmo-nature of this enzyme whilst its formalin sensitivity was shown by the fact that formalin fixation and even postfixation almost totally abolished its activity. Preganglionic nerve division had no effect on the Es nsE activity. Following postganglionic nerve division Es nsE activity was greatly diminished after three days but was fully restored two months after the operation.

EnnsE was only demonstrable in fixed sections indicating the histo-nature of the enzyme. The activity was mainly localized in cytoplasmic granules in all the neurones. After decentralization and axotomy EnnsE behaved in a similar way to EnnsE respectively.

**Phosphatases (Chapter II)** The distribution of acid phosphatase (AcPase) differed considerably in fresh and fixed sections. In the former the reaction product was diffuse in the ganglion cell cytoplasm and the interstitial tissue also showed some activity. In the latter the activity was mainly confined to cytoplasmic granules of the ganglion cells while the interstitium was inactive. Preganglionic denervation had no effect on the AcPase activity in the ganglion. After postganglionic denervation AcPase was much increased for about 30 days. The normal level was reached in two months.

Alkaline phosphatase (AlPase) was mainly localized in the capillaries. Neither pre nor postganglionic nerve division had any effect on the activity of this enzyme.

Adenosine triphosphatase (ATPase) exhibited intense activity in the capillaries and nerve fibres. The satellite cells and Schwann cell and possibly also the cell membrane of the neurones showed activity. After decentralization the activity was not appreciably changed while after axotomy the interstitial activity except that of the capillaries was greatly decreased in a few days. Normal values were reached in 60 days. The activity of the ganglion cells did not show any change after axotomy.

**Oxidative Enzymes (Chapter III)** The histochemically demonstrated oxidative enzymes (NADH, NADPH, succinate,  $\beta$  hydroxybutyrate, isocitrate, malate, lactate,  $\alpha$  glycerophosphate, menadione, glutamate and glucose 6 phosphate tetrazolium reductases, cytochrome oxidase and monoamine oxidase) exhibited more intense activity in the cytoplasm of the ganglion cells than in the surrounding satellite cells while the other interstitial structures showed a still weaker activity than the latter. The distribution of the cytoplasmic activity was partly granular and partly diffuse. Alcohol dehydrogenase was inactive in the whole ganglion.

Preganglionic nerve division had no effect on the activities of the oxidative enzymes in the whole ganglion. After postganglionic nerve division the activities of these dehydrogenases increased returning to normal again about 60 days after the operation. After the initial increase the activity of succinate dehydrogenase began to decrease on about the 10th day after axotomy. Normalization was complete in 60 days. Cytochrome oxidase and monoamine oxidase activities decreased after axotomy the changes being reversible as usual.

**Catecholamines (Chapter IV)** A weak to moderate green fluorescence localized to the synaptic terminals was seen to surround nearly all the ganglion cell bodies after treatment of the freeze dried ganglia with formaldehyde vapour. The fluorescence was intensified after successive injections of monoamine oxidase inhibitor (malamide) and noradrenaline.

The extirpation of the superior cervical ganglion had no effect on any enzyme activities of the ipsilateral ciliary ganglion of the rat.



## REFERENCES

- ADAMUK *Medic Centralbl* 1870 1<sup>o</sup> 179 Referred by Schwalbe 1879
- ALDRIDGE W N The differentiation of true and pseudo cholinesterase by organophosphorus compounds *Biochem J* 1953 53 62-67
- ALDRIDGE W N Serum esterases 1 Two types of esterase (A and B) hydrolysing p-nitrophenyl acetate propionate and butyrate and a method for their determination *Biochem J* 1953 a 53 110-117
- ALDRIDGE W N Some esterases of the rat *Biochem J* 1954 57 692-702
- ALDRIDGE W N and A N DAVISON The inhibition of erythrocyte cholinesterase by triesters of phosphate acids 1 Diethyl p nitrophenyl phosphate (E 600) and analogues *Biochem J* 1952 57 67-70
- ALDRIDGE W N and M K JOHNSON Cholinesterase succinic dehydrogenase nucleic acids esterase and glutathione reductase in sub cellular fractions from rat brain *Biochem J* 1959 73 270-276
- ALLES G A and R C HAWES Cholinesterases in the blood of man *J Biol Chem* 1940 133 375-390
- ANDERSON P J and S K SONG Acid phosphatase in the nervous system *J Neuro path exp Neurol* 1962 21 263-283
- ARIOKA I and H TANIMUKAI Histochemical studies on monoamine oxidase in the midbrain of the mouse *J Neurochem* 1957 1 311-315
- AUGUSTINSSON K -B Cholinesterases A study in comparative enzymology *Acta physiol scand* 1948 15 Suppl 52 1-182
- AUGUSTINSSON K -B and B NACHMANSOHN Distinction between acetylcholine esterase and other choline ester splitting enzymes *Science* 1949 110 98-99
- AUSTIN L and W K BERRY Two selective inhibitors of cholinesterase *Biochem J* 1953 54 695-700
- BANNISTER R G and F C A ROMANUL The localization of alkaline phosphatase activity in cerebral blood vessels *J Neurol Neurosurg Psychiat* 1963 26 333-340
- BARKA T Cellular localization of acid phosphatase activity *J Histochem Cytochem* 1962 10 231-232
- BARNETT R J The distribution of esterolytic activity in the tissues of the albino rat as demonstrated with indoxyl acetate *Anat Rec* 1952 114 577-600
- BARNETT R J and A M SELIGMAN Histochemical demonstration of esterases by production of indigo *Science* 1951 114 579-582
- BARRON K D and H SKLAR Response of lysosomes of bulbospinal motoneurons to axon section. *Neurology* 1961 11 866-875
- BARRON K D and T O TUACBAY Histochemistry of acid phosphatase and thiamine pyrophosphatase during axon reaction *Amer J Path* 1962 40 637-652
- BAYLISS H J and A TODRICK The use of a selective acetylcholinesterase inhibitor in the estimation of pseudocholinesterase activity in rat brain *Biochem J* 1956 62 62-67
- BECKER N H S GOLDFISCHER W -Y SHIN and A B NOVIKOFF Localization of enzyme activities in the rat brain *J biophys biochem Cytol* 1960 8 649-663
- BERGMANN N F and S RIMON The effect of pH variations on the activities of C-esterase *Biochem J* 1958 70 339-344
- BERGMANN N F R SEGAL and S RIMON, A new type of esterase in hog kidney extract *Biochem J* 1957 67 481-486
- BERNSOHN J I K D BARRON and A R HESS Multiple nature of acetylcholine esterase in nervous tissue *Nature (Lond)* 1962 195 285-286
- BIRKS R and F C MACINTOSH Acetylcholine metabolism of a sympathetic ganglion *Canad J Biochem* 1961 39 787-827



- BITSNIKY I The reversible activation of lysosomes in normal cells and the effects of pathological conditions. In *Ciba Foundation Symposium Lysosomes* Churchill London 1963 362-373
- BLASCHKO H J M HAGER and I HAGER Mitochondrial enzymes and chromatin granules *J Physiol (Lond)* 1957 137 316-322
- BODIAN D and H C MELLORE The regenerative cycle of motoneurons with special reference to phosphatase activity *J exp Med* 1945 81 461-487
- BONDAREFF W Submicroscopic morphology of granular vesicles in sympathetic nerves of rat pineal body *Z Zellforsch* 1963 67 211-218
- BRATTGARD S -O J -I EMDRÖM and H HEDEN The chemical changes in regenerating neurons *J Neurochem* 1957 1 316-323
- BRODAL A Anterograde and retrograde degeneration of nerve cells in the central nervous system To be published
- BRODY I M R H I WANG and J A BAIN Intracellular distribution of diphosphopyridine nucleotide-cytochrome c reductase and cytochrome c oxidase in mammalian brain *J Biol Chem* 1952 198 821-826
- BROWN I M Cholinesterases in the superior cervical ganglion of the rat after preganglionic denervation and axotomy *J Physiol (Lond)* 1958 117 71-89
- BROWN G I H McLENNAN and J I LAROC Acetylcholine metabolism of axotomized sympathetic ganglia *J Physiol (Lond)* 1952 118 601
- BROWN G L and J I LAROC The effect of degenerative section of ganglionic axons on transmission through the ganglion *J Physiol (Lond)* 1951 113 565-583
- BRÜCKE I Ph The cholinesterases in sympathetic ganglia *J Physiol (Lond)* 1937 89 429-437
- BURKE J D O SOLNYTSKY and C E MEYER Acid phosphatase reaction during the regenerative cycle of motor neurons in cats and domestic fowl after interruption of the sciatic nerve *Bull Georgetown Univ Med Cent* 1949 3 51-68
- BURN J H and M J RAND Sympathetic postganglionic cholinergic fibres *Brit J Pharmacol* 1960 15 56-66
- BURN J H and M J RAND Acetylcholine in adrenergic transmission *Ann Rev Pharmacol* 1965 5 163-181
- BURSTONE M S The cytochemical localization of esterase *J nat Cancer Inst* 1957 18 167-172
- BURSTONE M S New histochemical techniques for the demonstration of tissue oxidase (cytochrome oxidase) *J Histochem Cytochem* 1959 7 112-122
- BURSTONE M S Histochemical demonstration of cytochrome oxidase with new amine reagents *J Histochem Cytochem* 1960 8 63-70
- BURSTONE M S Modifications of histochemical techniques for the demonstration of cytochrome oxidase *J Histochem Cytochem* 1961 9 59-63
- CAUNA N and N T NAIK The distribution of cholinesterases in the sensory ganglia of some mammals *J Histochem Cytochem* 1963 11 129-138
- CAUNA N N T NAIK D H LEAMING and P ALBERTI The distribution of cholinesterases in the autonomic ganglia of man and some mammals A preliminary report *Bibl anat (Basel)* 1961 2 90-96
- CAUSEY G and H HOFFMAN Cytoplasmic synthesis in nerve cells *Brit J Cancer* 1955 9 666-673
- CAVANAGH J H R S THOMPSON and G R WEBSTER The localization of pseudocholinesterase activity in nervous tissue *Quart J exp Physiol* 1954 39 185-197
- CHIACKO L W and J A CERY Histochemical localization of cholinesterase in the amphibian spinal cord and alterations following ventral root section. *J Anat (Lond)* 1960 94 71-81
- CLITHEROW J W M MITCHEARD and N J HARPER The possible biological function of pseudocholinesterase *Nature (Lond)* 1963 199 1000-1001
- COIMBRA A and A S TAVARES Desoxyribonuclease II and ribonuclease and acid phosphatase activities in normal and chromatolytic neurons Histochemical and photometric study *Histochemie* 1964 3 509-520
- CORRODI H and N -Å HILLARP Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 2 Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 5,7 Dimethoxyisochinolinderivaten und Formaldehyd *Helv chim Acta* 1963 46 2425-2430

- CORRODI, H and A -Å HILLARP, Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 2. Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd *Helv chim Acta* 1964 47 911-918
- COLPLAND R E and R L HOLMES The use of cholinesterase techniques for the demonstration of peripheral nervous structures *Quart J micr Sci* 1957 98 327-330
- CROOK, J C Acetylcholinesterase activity of capillary blood vessels in the central nervous system of the rabbit *Nature (Lond)* 1963 199 41-43
- CSILLIK B and G B KOELLE Histochemistry of the adrenergic and the cholinergic autonomic innervation apparatus as represented by rat iris *Acta histochem (Jena)* 1965 22 350-363
- CSILLIK B and G SAVAY Contributions to the histochemistry of cholinesterase activity in the nervous system *Acta morph Acad Sci hung* 1954 4 103-109
- CUMMINS J and H HÄDEN Adenosine triphosphate levels and adenosine triphosphatases in neurons, glia and neuronal membranes in the vestibular nucleus *Biochim biophys Acta (Amst)* 1962 60 271-283
- DAHLSTRÖM A and K FUXE Evidence for the existence of monoamine neurons in the central nervous system *Acta physiol scand* 1965 64 Suppl 247 1-85
- DEANE H W Nuclear location of phosphatase activity: fact or artifact? *J Histochem Cytochem* 1963 11 443-444
- DE DUVE C The lysosome concept. In *Ciba Foundation Symposium: Lysosomes* Churchill London, 1963 1-31
- DE DUVE C, B C PRESSMAN, R GIANETTO, R WATTIAUX and F APPELMANS Tissue fractionation studies 6. Intracellular distribution patterns of enzymes in rat liver tissue *Biochem J* 1955 60 604-617
- DE LORENZO, A J The fine structure of synapses in the ciliary ganglion of the chick *J biophys biochem Cytol* 1960 7 31-36
- DE ROBERTIS E Submicroscopic changes of the synapse after nerve section in the acoustic ganglion of the guinea pig. An electron microscope study *J biophys biochem Cytol* 1956 2 503-512
- DE ROBERTIS E and A PELLEGRINO DE IRALDI A plurivesicular component in adrenergic nerve endings *Anat Rec* 1961 129 299
- DE ROBERTIS F A PELLEGRINO DE IRALDI G RODRIGUEZ DE LORES ARNAIZ and J C CONEZ On the isolation of nerve endings and synaptic vesicles *J biophys biochem Cytol* 1961 9 229-235
- DE ROBERTIS E A PELLEGRINO DE IRALDI G RODRIGUEZ DE LORES ARNAIZ and L SALGANICOFF Cholinergic and non cholinergic nerve endings in rat brain I. Isolation and subcellular distribution of acetylcholine and acetylcholinesterase *J Neurochem* 1962 9 23-36
- DE ROBERTIS E G RODRIGUEZ DE LORES ARNAIZ L SALGANICOFF A PELLEGRINO DE IRALDI and L M ZIEHER Isolation of synaptic vesicles and structural organization of the acetylcholine system within brain nerve endings. *J Neurochem* 1963 10 225-236
- DHAR S K Cholinesterase in decentralized and axotomized sympathetic ganglia *J Physiol (Lond)* 1958 144 27P-28P
- DIEGENBACH P C Use of inhibitors in cholinesterase histochemistry *Nature (Lond)* 1965 207 308
- EASON L H and E STEDMAN The specificity of choline-esterase *Biochem J* 1957 51 1723 1729
- EDER M Der histochemische Nachweis des Fermentes Monoaminoxidase. Entwicklung einer Nachweismethode und deren Anwendung *Beitr path Anat* 1957 117 343 396
- ENINGER B Ocular and orbital vegetative nerves *Acta physiol scand* 1966 67 Suppl 268 1-35
- ENINGER B and B FALLK Noradrenaline and cholinesterases in concomitant nerve fibres in the rat iris *Life Sciences* 1965 4 2037-2100
- ERANKO O Histochemical evidence of intense phosphatase activity in the hypothalamic magnocellular nuclei of the rat *Acta physiol scand* 1951 24 1-6
- ERANKO O On the histochemistry of the adrenal medulla of the rat with special reference to acid phosphatase *Acta anat (Basel)* 1952 16 Suppl 17 1-60

- FRÄNKÖ O Quenching of tissue for freeze-drying *Acta anat (Basel)* 1951 77 331-336
- FRÄNKÖ O Distribution of fluorescent islets adrenaline and noradrenaline in the adrenal medulla of the hamster *Acta endoc (Abh)* 1955 18 174-179
- FRÄNKÖ O Fluorescent islets adrenaline and noradrenaline in the adrenal medulla of some common laboratory animals *Ann Med exp Fenn* 1955 a 53 278-290
- FRÄNKÖ O Specific demonstration of acetylcholinesterase and non-specific cholinesterase in the adrenal gland of the rat *Histochemie* 1959 1 257-267
- FRÄNKÖ O Personal communication to Dr FALCK acknowledged in FALCK and TORP (1961)
- FRÄNKÖ O Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour *J Histochem Cytochem* 1964 12 487-489
- FRÄNKÖ O Histochemistry of nervous tissues Catecholamines and cholinesterases 1967 to be published
- FRÄNKÖ O and M HÄRKÖNEN Histochemical demonstration of biogenic amines in the cytoplasm of sympathetic ganglion cells of the rat *Acta physiol scand* 1963 58 285-286
- FRÄNKÖ O and M HÄRKÖNEN Effect of axon division on the distribution of noradrenaline and acetylcholinesterase in the sympathetic neurons of the rat *Acta physiol scand* 1965 63 411-412
- FRÄNKÖ O M HÄRKÖNEN A KOKKO and L RÄINÄNEN Histochemical and starch gel electrophoretic characterization of desmo- and lysoesterases in the sympathetic and spinal ganglia of the rat *J Histochem Cytochem* 1964 12 570-581
- FRÄNKÖ O A KOKKO and U SÖDERHOLM Histochemical evidence of three types of esterase in the adrenal medulla of the rat *Histochemie* 1967 2 383-388
- FRÄNKÖ O and L RÄINÄNEN Fibres containing both noradrenaline and acetylcholinesterase in the nerve net of the rat iris *Acta physiol scand* 1965 63 505-506
- FRIKÅ R Histochemical and electrophoretic properties of cholinesterases and non specific esterases in the retina of some mammals including man *Acta ophthal (Abh)* 1963 Suppl 77 1-113
- FALCK B Observations on the possibilities of the cellular localization of monoamines by a fluorescence method *Acta physiol scand* 1962 36 Suppl 197 1-25
- FALCK B N -Å HILLARP C LILJENF and Å TORP Fluorescence of catechol amines and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962 10 318-351
- FALCK B and CH OWMAN A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines *Acta Univ Lund* 1965 7 1-23
- FALCK B and Å TORP A fluorescence method for histochemical demonstration of noradrenaline in the adrenal medulla *Med exp (Basel)* 1961 5 429-432
- FERGUSON M M Observations on the histochemical distribution of alcohol dehydrogenase *Quart J micr Sci* 1965 106 289-297
- FISCHER C and S SORIANI Enzymic activities in the spinal cord after sciatic section Alkaline and acid phosphatases 5 nucleotidase and ATPase *J Neurochem* 1959 4 71-77
- FISHER A W F Acid phosphatase activity in the inferior vagal ganglion *J Anat (Lond)* 1965 99 925
- FISHER J and V MALIK A histochemical study on the effect of transection of the facialis nerve on the picture of glycerophosphate lactate succinate malate and glutamate dehydrogenase in the cells of the facial nerve nucleus *Acta histochem (Jena)* 1964 19 369-376
- FISHER A W F and S D SUTHERLAND Indoxyl esterase in the nervous system of the monkey *J Anat (Lond)* 1965 99 215-217
- FISHER A W F and S D SUTHERLAND Organophosphate resistant esterase acid phosphatase and  $\beta$  galactosidase activities in the inferior vagal (nodose) ganglion and dorsal vagal nucleus of the monkey following cervical vagotomy *J Anat (Lond)* 1965 a 99 585-593
- FISHMAN J E and M HAYASHI Enzymomorphology of rat brain  $\beta$  glucuronidase alkaline phosphatase esterase *J Histochem Cytochem* 1962 10 515-519
- GOLDES F F E K ZSIGMOND V M FOIDES and F G ERDŐS The distribution of

- acetylcholinesterase and butyrylcholinesterase in the human brain *J Neurochem* 1962 9 559-572
- FREDRICKSON B and F SJOQUIST A cytomorphological study of cholinesterase in sympathetic ganglia of the cat *Acta morph neerl-scand* 1962 5 140-166
- FRIEDE R L Transport of oxidative enzymes in nerve fibres a histochemical investigation of the regenerative cycle in neurons *Exp Neurol* 1959 1 441-446
- FRIEDE R L, Histochemical investigations on succinic dehydrogenase in the central nervous system IV A histochemical mapping of the cerebral cortex of the guinea pig *J Neurochem* 1960 5 16-171
- FRIEDE R L and I M FLEWING A mapping of oxidative enzymes in human brain *J Neurochem* 1962 9 179-188
- FRIEDE R L and R A LAY Mitochondria and mitochondrial enzymes A comparative study of localization in the cat's brain stem *Histochemie* 1961 2 186-191
- FUKUDA T Application of the methylene green pyronin (Brachet's test) onto the thiocholine method for histochemical demonstration of neuronal acetylcholinesterase *Bull Osaka med Sch* 1959 5 46-54
- FLUXUS T and G B KOELLE The cytochemical localization of intracellular neuronal acetylcholinesterase *J biophys biochem Cytol* 1959 4 433-440
- GEDIGK P and E BONTJE Über den Nachweis von hydrolytischen Enzymen in Lipopigmenten *Z Zellforsch* 1956 44 495-518
- GERBZDOWSKI A *Cholinesterases A Histochemical Contribution to the Solution of Some Functional Problems Modern Trends in Physiological Sciences Vol 3 Pergamon Press London, Oxford New York Paris* 1959
- GERMAIN M and P PROLLY Adenosinetriphosphatase activity in synaptic vesicles of rat brain *Biochem Pharmacol* 1965 14 1815-1819
- GIACOBINI E Histochemical demonstration of acetylcholinesterase activity in isolated nerve cells *Acta physiol scand* 1956 36 176-290
- GIACOBINI E The intracellular distribution of cholinesterase in the nerve cell studied by means of a quantitative micro-method *Acta physiol scand* 1957 4 Suppl 145 49-50
- GIACOBINI E The distribution and localization of cholinesterases in nerve cells *Acta physiol scand* 1959 42 Suppl 156 1-45
- GIACOBINI E Quantitative determination of cholinesterase in individual spinal ganglion cells *Acta physiol scand* 1959 42 238-254
- GIACOBINI E The intracellular localization of cholinesterase *J Histochem Cytochem* 1960 8 419-424
- GIACOBINI E and B HOLMSTEDT Cholinesterase content of certain regions of the spinal cord as judged by histochemical and Cartesian diver technique *Acta physiol scand* 1958 42 12-27
- GLENNER G G H J BLUTNER and G W BROWN The histochemical demonstration of monoamine oxidase activity by tetrazolium salts *J Histochem Cytochem* 1957 5 591-600
- GOLDFISCHER S E PISNER and A B NOVIKOFF The localization of phosphatase activities at the level of ultrastructure *J Histochem Cytochem* 1964 12 72-95
- GOMORI G *Microscopic Histochemistry Principles and Practice The University of Chicago Press Chicago* 1952
- GOMORI G Human esterases *J Lab and Clin Med* 1953 42 443-453
- GOMORI G Histochemistry of human esterases *J Histochem Cytochem* 1955 3 479-484
- GRAY E G and V F WHITTAKER The isolation of synaptic vesicles from the central nervous system *J Physiol (Lond)* 1960 153 35P-37P
- GROMADZKI L G and G B KOELLE The effect of axotomy on the acetylcholinesterase of the superior cervical ganglion of the cat *Biochem Pharmacol* 1965 14 1745-1754
- GROSSER W Histochemischer Nachweis hydrolytischer Enzyme mit Hilfe der Azofarbstoffmethode Untersuchungen zur Methodik und vergleichenden Histotopik der Esterasen und Phosphatasen bei Wirbeltieren *Histochemie* 1958 1 48-96
- HAMBY R L Isolation of intracellular acid cycle intermediates by nerve cell body dissection *J Neurochem* 1961 8 31-35
- HAMBY R L and J MALMORS and CH SACHS Standardization of paraformaldehyde

- and of certain procedures for the histochemical demonstration of catecholamines *J Histochem Cytochem* 1964 13 147
- HAMBERGER B and K -A NORBERG Monamines in sympathetic ganglia studied with fluorescence microscopy *Experientia (Basel)* 1963 8 31-35
- HAMBERGER B and K -A NORBERG Adrenergic synaptic terminals and nerve cells in bladder ganglia of the cat *Int J Neuroparmacol* 1965 4 41-45
- HAMBERGER B K -A NORBERG and F Sjöqvist Cellular localization of monoamines in sympathetic ganglia of the cat *Life Sciences* 1963 1 659-661
- HAMBERGER B K -A NORBERG and U UNGERSTEDT Adrenergic synaptic terminals in autonomic ganglia *Acta physiol scand* 1965 64 285-288
- HANNIBAL M J and M M NACHIAS Further studies on the lyso- and dehydrogenase components of several hydrolytic enzymes and their histochemical significance *J biophys biochem Cytol* 1959 5 259-288
- HANSHAL J M M NACHIAS and A M SELIGMAN Histochemical localization of cytochrome oxidase in human tissues and neoplasms *Cancer* 1960 13 1008-1016
- HANSON V and G TOSCHI Electron microscopy of microsomal fraction from rat brain *Exp Cell Res* 1959 16 256-271
- HARD W I and A G. IFFERSON Studies on the histochemical localization of cholinesterase in nerve tissue of the dog *Anat Rec* 1949 105 494-495
- HARTMANN I J Electron microscopy of motor nerve cells following section of axones *Anat Rec* 1954 118 19-34
- HASHIMOTO T J S KALLZA and M S BERTSON The effect of menadione and phenazine methosulfate on the tetrazolium reduction system under histochemical conditions *J Histochem Cytochem* 1963 12 797-804
- HESS R and A G F PEARSE The histochemistry of indoxyl esterase of rat kidney with special reference to its cathepsin like activity *Brit J exp Path* 1958 39 292-299
- HESS R and A G F PEARSE Histochemical and homogenization studies of mitochondrial glycerophosphate dehydrogenase in the nervous system *Nature (Lond)* 1961 191 718-719
- HESS R D C SCARPELLI and A G F PEARSE The cytochemical localization of oxidative enzymes II Pyridine nucleotide linked dehydrogenases *J biophys biochem Cytol* 1958 4 11 753-760
- HORRIGER F F The hydrolysis of indoxyl esters by rat esterases. *Biochem J* 1957 67 600-607
- HOGBOOM G H and W C SCHNEIDER Cytochemical studies IV Physical state of certain respiratory enzymes of mitochondria *J Biol Chem* 1952 194 513-519
- HOLMSTEDT B A modification of the thiocholine method for the determination of cholinesterase II Histochemical application. *Acta physiol scand* 1957 40 331-337
- HOLMSTEDT B G LUNDGREN and F Sjöqvist Determination of acetylcholinesterase activity in normal and denervated sympathetic ganglia of the cat A biochemical and histochemical comparison. *Acta physiol scand* 1963 57 235-247
- HOLMSTEDT B and F Sjöqvist Distribution of acetylcholinesterase in the ganglion cells of various sympathetic ganglia *Acta physiol scand* 1959 47 284-296
- HOLMSTEDT B and F Sjöqvist Pharmacological properties of  $\gamma$ -aminobutyrylcholine A supposed inhibitory transmitter *Biochem Pharmacol* 1960 3 297-304
- HOLMSTEDT B and F Sjöqvist Some principles about histochemistry of cholinesterase with special reference to the thiocholine method *Bibl anat (Basel)* 1961 7 1-10
- HOLMSTEDT B and G TOSCHI Enzymic properties of cholinesterases in subcellular fractions from rat brain *Acta physiol scand* 1959 47 280-283
- HOLT S J A new principle for the histochemical localization of hydrolytic enzymes *Nature (Lond)* 1952 169 271-273
- HOLT S J and R F J WITHERS Cytochemical localization of esterases using indoxyl derivatives *Nature (Lond)* 1952 169 1012-1014
- HOPPU V K and C G GLENNER Further observations on histochemical esterase and amidase activities with similarities to trypsin *J Histochem Cytochem* 1963 11 520-528

- OPSU V K and G G GLENNER Enzymes hydrolysing acetyl naphthylamides  
*J Histochem Cytochem* 1964 12 36-37
- OWE H A and J B FLEKNER Succinic dehydrogenase in regenerating neurons  
*J Biol Chem* 1947 167 663-671
- OWE H A and R G MELLORS Cytochrome oxidase in normal and regenerating neurons  
*J exp Med* 1945 81 489-500
- UDSON G A LAZAROW and J F HARTMANN, A quantitative electron microscopic study of mitochondria in motor neurones following axonal section  
*Exp Cell Res* 1961 24 440-456
- JOHNS A A demonstration of dehydrogenase activity in the mitochondria of living cells  
*Quart J micr Sci* 1956 97 165-170
- UNT C C and P G NELSON Structural and functional changes in the frog sympathetic ganglion following cutting of the presynaptic nerve fibres  
*J Physiol (Lond)* 1965 177 1-20
- YDEN H The neuron In *The Cell Vol II* Academic Press New York London 1960 215-323
- YDEN H The neuron and its glia a biochemical and functional unit  
*Endeavour* 1962 21 144-155
- YDEN H ■ LOVTRUP and A PIGON Cytochrome oxidase and succinoxidase activities in the spinal ganglion cells and in glial capsule cells  
*J Neurochem* 1958 2 304-311
- YDEN H and A PIGON A cytophysiological study of the functional relationship between oligodendroglial cells and nerve cells of Deiters' nucleus  
*J Neurochem* 1960 6 57-72
- ARKONEN M Carboxylic esterases oxidative enzymes and catecholamines in the superior cervical ganglion of the rat and the effect of pre and postganglionic nerve division  
*Acta physiol scand* 1964 63 Suppl 237 1-94
- ELIS L Spinal cord synapses in the cat The normal appearances by light microscope  
*Brain* 1964 87 543-553
- AWAI H Cytoplasmic granules in nerve cells of the spinal cord following transection of sciatic nerve of guinea pig  
*Exp Neurol* 1963 7 457-463
- AWAKITA Y Colorimetric estimation of succinic dehydrogenase activity in the rabbit brain with tetrazolium salt  
*Acta psychiat (Pbh)* 1956 31 459-465
- LEIN H Der Nachweis einer oxydativen Stoffwechselsteigerung in der »primär gereizten« Spinalganglienzelle am Verhalten der Bernsteinsäuredehydrogenase  
*Z ges Neurol Psychiat* 1960 201 81-96
- LINGMAN G I and J D KLINGMAN Monoamine oxidase activity in peripheral organs and adrenergic tissues of the rat  
*Biochem Pharmacol* 1966 15 77-91
- OELE G B The histochemical differentiation of types of cholinesterases and their localizations in tissues of the cat  
*J Pharmacol exp Ther* 1950 100 158-179
- OELE G B The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions  
*J Pharmacol exp Ther* 1951 103 153-171
- OELE G B The histochemical localization of cholinesterases in the central nervous system of the rat  
*J comp Neurol* 1954 100 211-235
- OELE G B The histochemical identification of acetylcholinesterase in cholinergic, adrenergic and sensory neurons  
*J Pharmacol exp Ther* 1955 114 167-184
- OELE G B Cholinesterases of the central nervous system  
*J Neuropath exp Neurol* 1955 a 14 23-27
- OELE G B A new concept of the neurohumoral functions of acetylcholine and acetylcholinesterase  
*J Pharm Pharmacol* 1962 14 65-90
- OELE G B Cytological distributions and physiological functions of cholinesterases In *Cholinesterases and Anticholinesterase Agents Handbuch der Experimentellen Pharmacologie Vol 15* Springer Verlag Berlin Göttingen, Heidelberg 1963 189-298
- OELE G B and C FOROGLOU KERAMEOS Electron microscopic localization of cholinesterases in a sympathetic ganglion by a gold thiocetic acid method  
*Life Sciences* 1965 4 417-424
- OELE W A and G B OELE The localization of external or functional acetylcholinesterase at the synapses of autonomic ganglia  
*J Pharmacol exp Ther* 1959 196 1-8

- KOFLE G B and A de I Valk Physiological implications of the histochemical localization of monoamine oxidase *J Physiol (Lond)* 1964 117 431-447
- KOENIG H Histochemical distribution of brain gangliosides. Lysosomes as glycolipoprotein granules *Nature (Lond)* 1962 195 782-784
- KOENIG H The autofluorescence of lysosomes. Its value for the identification of lysosomal constituents *J Histochem Cytochem* 1963 11 546-557
- KOENIG H Histochemical study of lysosomes and lipofuscin granules in the nervous system *Anat Rec* 1963 148 303
- KOKKO A Histochemical and cytophotometric observations on esterases in the spinal ganglion of the rat *Acta physiol scand* 1963 67 Suppl 261 1-76
- KOPIN I J Storage and metabolism of catecholamines. The role of monoamine oxidase *Pharmacol Rev* 1964 16 179-191
- KRUTZBERG C W Changes of coenzyme (II N) diaphorase and II N linked dehydrogenase during axonal resection of the nerve cell *Nature (Lond)* 1963 99 393-394
- KUMAMOTO I and C H BOLKME Experimental studies on the oxidative enzymes and hydrolytic enzymes in spinal neurons. I A histochemical and biochemical investigation of the spinal ganglion and the spinal cord following sciatic neurotomy in the guinea pig *Acta anat (Basel)* 1963 55 255-277
- LAMPE A M and F D BLEKKE Acid phosphatase in peripheral degenerating and regenerating neurons *Anat Rec* 1947 97 395-396
- LAFFLE A C-N LIU and F W LAFFLE Acid phosphatase activity as related to nucleic acid sites in the nerve cell *Anat Rec* 1964 119 305-323
- LAZARUS S S and H BARDEN Histochemistry and electron microscopy of mitochondrial adenosinetriphosphatase *J Histochem Cytochem* 1962 10 285-293
- LENIHOSKA M A Das Ganglion ciliare der Vögel *Arch mikr Anat* 1911 76 745-769
- LEWIS P R The histochemical location of cholinesterases *J Physiol (Lond)* 1965 181 231-241P
- LEWIS I R A B LINE and C C D SILVER The electron microscopic distribution of cholinesterase in rat brain *J Physiol (Lond)* 1965 181 151
- LEWIS I R and C C D SILVER Demonstration of cholinesterase activity with electron microscope *J Physiol (Lond)* 1964 175 51-71
- LEWIS I R and C C D SILVER Electron microscopic distribution of cholinesterase in cholinergic neurones *J Anat (Lond)* 1965 99 941
- LEWIS I R and C C D SILVER Changes in cholinesterase activity following axotomy *J Physiol (Lond)* 1965 178 25P-26P
- LIEN L La recherche histochimique des phosphatases Etude critique *Bull Histol Techn Micro* 1948 23-31
- LOWRY O H A R ROBERTS K A LEINER M-I WE A I FARR and R W ALBERS The quantitative histochemistry of brain III Ammon's Horn *J Biol Chem* 1951 197 39-49
- MARNETT C C and R L HUNTER The distribution of esterases in mouse tissues *J Histochem Cytochem* 1959 7 42-49
- MARTIN A R and G PILAR Dual mode of synaptic transmission in the avian ciliary ganglion *J Physiol (Lond)* 1963 168 443-463
- MCCURKIN I T A method for the cytochemical demonstration of sodium activated adenosine triphosphatase *J Histochem Cytochem* 1964 12 654-658
- MELSAAC R J and C B KOFLE Comparison of the effects of inhibition of external internal and total acetylcholinesterase upon ganglionic transmission *J Pharmacol exp Ther* 1959 126 9-20
- MCLENNAN H Acetylcholine metabolism of normal and axotomized ganglia *J Physiol (Lond)* 1954 124 113-116
- MEATH J A and A POPE Histochemical distribution of indophenol oxidase and acid phosphatase in rat cortex *Fed Proc* 1950 9 204
- MENDEL B D K MYERS I N ULLDERT A C RUS and W M DE BRUIJN Aliesterase inhibitors and growth *Brit J Pharmacol* 1963 8 217-224
- MUSTAKALLIO K K Histochemical demonstration of succinic dehydrogenase activity in human brain *Ann Med exp Fenn* 1964 32 175-177
- MYERS D K and B MENDEL Studies on aliesterase and other lipid hydrolysing enzymes *Biochem J* 1953 53 16-25

- MYERS D H and E C SLATER The enzymic hydrolysis of adenosine triphosphate by liver mitochondria I Activities at different pH values *Biochem J* 1957 67 558-572
- NACHLAS M M W PRINZ and A M SELIGMAN Quantitative estimations of lyso- and desmoenzymes in tissue sections with and without fixation *J biophys biochem Cytol* 1956 2 487-502
- NACHLAS M M and A M SELIGMAN The comparative distribution of esterase in the tissues of five mammals by a histochemical technique *Anal Rec* 1949 105 677-696
- NACHLAS M M A -C FSOU E DE SOUZA C -S CHENG and A M SELIGMAN Cytochemical demonstration of succinic dehydrogenase by the use of a new p nitrophenyl substituted ditetrazole *J Histochem Cytochem* 1957 5 420-436
- NACHMANSOHN D *Chemical and Molecular Basis of Nerve Activity* Academic Press New York and London 1959
- NACHMANSOHN D and E C HOFF Effects of dorsal root section on choline esterase concentration in spinal cord of cats *J Neurophysiol* 1944 7 27-36
- NAIDOO D Adenosine triphosphatase A neurohistochemical study *J Histochem Cytochem* 1962 10 731-740
- NANDY K and G H BOURNE Alkaline phosphatases in brain and spinal cord *Nature (Lond)* 1963 200 1216-1217
- NANDY K and G H BOURNE Adenosine triphosphatase and 5 nucleotidase in spinal cord *Arch Neurol* 1964 11 547-553
- NANDY K and G H BOURNE A histochemical study of the localization of the oxidative enzymes in the neurones of the spinal cord in rats *J Anat (Lond)* 1964 a 98 647-653
- NANDY K and G H BOURNE A histochemical study of the localization of succinic dehydrogenase cytochrome oxidase and diphosphopyridine nucleotide diaphorase in the synaptic regions of the spinal cord in the rat *J Histochem Cytochem* 1964 b 12 188-193
- NATHAN P and M H APRISON Cholinesterase activity in cytoplasmic particles from rabbit brain *Fed Proc* 1955 14 106-107
- NEWMAN W E A KABAT and A WOLF Histochemical studies on tissue enzymes V A difficulty in enzyme localization in the acid range due to selective affinity of certain tissues for lead its dependence on pH *Amer J Path* 1950 26 489-503
- NIEMI M N SCHOR and A G E PEARSE Effects of dehydration on the mitochondria of the mouse kidney *Nature (Lond)* 1960 185 786-787
- NORBERG K A Adrenergic innervation of the intestinal wall studied by fluorescence microscopy *Int J Neuropharmacol* 1964 3 379-382
- NOVIKOFF A B Lysosomes and related particles In *The Cell Vol II* Academic Press New York London 1961 423-488
- NOVIKOFF A B Lysosomes in the physiology and pathology of cells Contribution of staining methods In *Ciba Foundation Symposium Lysosomes* Churchill London 1963 36-77
- NOVIKOFF A B and E ESSNER Pathological changes in cytoplasmic organelles *Fed Proc* 1962 21 1130-1142
- NOVIKOFF A B W -Y SHIN and J DRUCKER Cold acetone fixation for enzyme localization in frozen sections *J Histochem Cytochem* 1960 8 37-40
- OGAWA K and R J BARNETT Electron cytochemical studies of succinic dehydrogenase and dihydronicotinamide adenine dinucleotide diaphorase activities *J Ultrastruct Res* 1965 12 488-508
- OKAKA S M YOSHIKAWA M UONO T MURO A IGATA H TANABE S UEDA and M TOMONAGA Über die Cholinesterase des autonomen und peripheren Nervensystems *Acta neuroveg (Wien)* 1963 20 249-264
- ORD M G and R H S THOMPSON Pseudo cholinesterase activity in the central nervous system *Biochem J* 1952 51 245-251
- OSINCHAK J Acid phosphatase activity and the identity of intracellular granules in neurosecretory cells of the rat *J Cell Biol* 1963 19 54A
- PADYKULA H A The localization of succinic dehydrogenase in tissue sections of the rat *Amer J Anat* 1952 91 107-145



- PALAY S I and G F PALADY The fine structure of neurons *J biophys biochem Cytol* 1955 1 69-88
- PEARSE A G L The esterases of the hypophysis and their functional significance *J Path Bact* 1956 72 171-487
- PEARSE A G L In *Histochemistry Theoretical and Applied* Churchill London 1960
- PEARSE A G L and R HESS Substantivity and other factors responsible for formalin patterns in dehydrogenase histochemistry *Experientia (Basel)* 1961 17 136-141
- PEARSE A G L, D C SCARFILL and S BROWN Cytochemical demonstration of the relationships between the DPN-diphosphorase and the succinic dehydrogenase systems *Biochem J* 1958 68 181
- PEARSON B and V DEYENDI A comparison between the histochemical demonstration of non specific esterase activity by 5 bromo-indoxyl acetate  $\alpha$  naphthyl acetate and naphthol AS acetate *J Histochem Cytochem* 1957 5 72-83
- PELEGRIANO DE IKALDI A, H LARINI DUGGAN and F DE ROBERTIS Adrenergic synaptic vesicles in the anterior hypothalamus of the rat *Anat Rec* 1963 145 521-531
- PEPLER W J and A G L PEARSE The histochemistry of the esterases of rat brain, with special reference to those of the hypothalamic nuclei *J Neurochem* 1957 1 193-202
- PERRY W L M Transmission in autonomic ganglia *Brit med Bull* 1957 13 220-226
- PERRY W L M and J TALESNIA The role of acetylcholine in synaptic transmission at parasympathetic ganglia *J Physiol (Lond)* 1953 119 455-469
- PINES J -I Zur Morphologie des Ganglion cilare beim Menschen *Z mikr - anat Forsch* 1927 10 313-380
- PLANTAGDT H T *Maag en maagcarcinoom een histochemisch onderzoek* Batteljee & Terpstra Leiden 1961 1-146
- POTANOS J N A WOLF and D COFFIN Cytochemical localization of oxidative enzymes in the human nerve cells and neuroglia *J Neuropath exp Neurol* 1959 18 627-635
- RETZEL G Untersuchungen über die Nervenzellen der cerebropinalen Ganglien und übrigen peripherischen Kopfganglien *Anat Anz* 1834 9 633-637
- RICHTER D and I G CROFT Blood esterases *Biochem J* 1912 36 746-757
- ROBINS I and D F SMITH A quantitative histochemical study of eight enzymes of the cerebellar cortex and subjacent white matter in the monkey *Res Publ Ass nerv ment Dis* 1953 39 305-327
- RODRIGUEZ DE LORES ARNAIZ G Subcellular localization of cholinesterase in brain *J Histochem Cytochem* 1964 12 696-699
- RODRIGUEZ DE LORES ARNAIZ C and F D I DE ROBERTIS Cholinergic and non cholinergic nerve endings in the rat brain II Subcellular localization of monoamine oxidase and succinate dehydrogenase *J Neurochem* 1962 9 503-508
- ROIZIN L Oxidases and peroxidases of the central nervous system *J Neuropath exp Neurol* 1955 14 47-69
- ROMANUL F C A and R H COHEN A histochemical study of dehydrogenases in central and peripheral nervous systems *J Neuropath exp Neurol* 1960 19 135
- ROSA C G and K -C TSOU Use of tetrazolium compounds in oxidative enzyme histo and cytochemistry *Nature (Lond)* 1961 197 990-991
- RUBINSTEIN L J I KLAFTZ and J MIGUEL Histochemical observations on oxidative enzyme activity of glial cells in a local brain injury *J Neuropath exp Neurol* 1962 21 116-136
- SAMORAJSKI T The application of diphosphopyridine nucleotide diaphorase methods in a study of dorsal ganglia and spinal cord *J Neurochem* 1960 5 349-353
- SAMORAJSKI T and G R LITZ Phosphomonoesterase changes associated with spinal cord chromatolysis *Lab Invest* 1961 10 129-143
- SANDLER M and G H BOURNE Intranuclear histochemical localization of adenosine triphosphatase *J Histochem Cytochem* 1962 10 636
- SÁVAY Gy, H CSILLIK and O BONDRAV Die unspezifische Esterase-Aktivität der sensorischen und vegetativen Ganglien, *Acta morph Acad Sci hung* 1953 3 207-213

- SAWYER C H Cholinesterases in degenerating and regenerating peripheral nerves  
*Amer J Physiol* 1946 146 246-253
- SAWYER C H and W H HOLLINSHEAD Cholinesterases in sympathetic fibres and ganglia  
*J Neurophysiol* 1945 8 139-153
- SCARFELLI D G R HEYS and A G E PEARSE The cytochemical localization of oxidative enzymes I. Diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase  
*J biophys biochem Cytol* 1958 4 11 747-752
- SCARFELLI D C and A G E PEARSE Cytochemical localization of succinic dehydrogenase in mitochondria  
*Anat Rec* 1958 137 133-152
- SCHARF J -H *Sensible Ganglien Handbuch der Mikroskopischen Anatomie des Menschen* Vol 11/3 Springer Verlag Berlin Göttingen Heidelberg 1958
- SCHARF J -H and C P ROWE Zur Verteilung der Kohlenhydrate und einiger Fermente im Ganglion semilunare des Rindes  
*Acta histochem (Jena)* 1957 4 129-145
- SCHULZE W and A WOLLENBERGER Elektronenmikroskopischer Nachweis von mitochondrialer Adenosintriphosphataseaktivität in tierischen Geweben  
*Histochemie* 1965 5 417-429
- SCHWABE G, Ganglion oculomotorii  
*Jena Z Med Naturw* 1879 13 173-268
- SCHWARZACHER H C Der Cholinesterasegehalt motorischer Nervenzellen während der axonalen Reaktion  
*Acta anat (Basel)* 1958 32 51-65
- SELLINGER O J and J DE BALDIAN VERSTER An esterase of rat cerebral cortex acting on  $\alpha$ -nitrophenylacetate. Method of assay properties and intracellular distribution  
*Analyt Biochem* 1962 3 479-488
- SHARMA D Histochemistry of cytoplasmic granules in motor neurons of rat spinal cord  
*Anat Rec* 1964 148 33
- SHAW C R and A L KOPF On the identity of «succinic dehydrogenase»  
*J Histochem Cytochem* 1965 13 431-433
- SHIMIZU N and N MORIKAWA Histochemical studies of succinic dehydrogenase of the brain of mice rats guinea pigs and rabbits  
*J Histochem Cytochem* 1957 5 334-345
- SHIMIZU N N MORIKAWA and Y ISHII Histochemical studies of succinic dehydrogenase and cytochrome oxidase of the rabbit brain with special reference to the results in the paraventricular structures  
*J comp Neurol* 1957 109 1-22
- SHIMIZU N N MORIKAWA and M OKADA Histochemical studies of monoamine oxidase in the brain of rodents  
*Z Zellforsch* 1959 49 389-400
- SHUTE C C D and P R LEWIS Electron microscopy of cholinergic terminals and acetylcholinesterase containing neurones in the hippocampal formation of the rat  
*Z Zellforsch* 1966 69 334-343
- SIEKEVITZ P and M L WATSON Cytochemical studies of mitochondria II. Enzymes associated with a mitochondrial membrane fraction  
*J biophys biochem Cytol* 1956 2 653-669
- SJOQVIST I Morphological correlate to a cholinergic sympathetic function  
*Nature (Lond)* 1962 191 298
- SLAVICH E Confronti fra la morfologia di gangli del parasimpatico encefalico e del simpatico cervicale con speciale riguardo alla struttura del ganglio ciliare  
*Z Zellforsch* 1932 15 688-730
- SMITH W K The results of application of the «acid phosphatase» method to nervous tissue after formalin fixation  
*Anat Rec* 1948 102 523-532
- SMITH W K and C LUTTRELL The «acid» phosphatase content of normal autonomic neurons and its increase following section of the cell processes  
*Anat Rec* 1947 97 47b
- SNELL, R S The histochemical appearances of cholinesterase in the superior cervical sympathetic ganglion and the changes which occur after preganglionic nerve section  
*J Anat (Lond)* 1958 97 408-418
- SNELL R S The histochemical localization of cholinesterase in the central nervous system  
*Bibl anat (Basel)* 1961 2 50-58
- SOBEL H J and E AVKIN Localization of acid phosphatase activity in rat pancreatic acinar cells. A light and electron microscopic study  
*J Histochem Cytochem* 1965 13 301-303
- STROMINGER J L and O H LOWRY The quantitative histochemistry of brain IV

- Lactic malic and glutamic dehydrogenases *J biol Chem* 1955 213 635-671
- SUMNER R J and K MYRBACK *The Enzymes* Academic Press New York, 1950 3 III 443-471
- SZENTÁGOOTAI J Zum elementaren Bau der interneuronalen Synapse *Acta anat (Basel)* 1957 30 827-811
- SZENTÁGOOTAI J, A DONHOFFER and K RAJKOVITS Die Lokalisation der Cholinesterase in der interneuronalen Synapse *Acta histochem (Jena)* 1955 1 272-281
- SÖDERHOLM U Histochemical localization of esterases, phosphatases and tetrazolium reductases in the motor neurones of the spinal cord of the rat and the effect of nerve division *Acta physiol scand* 1965 67 Suppl 256 1-60
- TAKAHASHI K and K HAMA Some observations on the fine structure of the synaptic area in the ciliary ganglion of the chick *Z Zellforsch* 1965 67 174-184
- TAKAHASHI K and K HAMA Some observations on the fine structure of nerve cell bodies and their satellite cells in the ciliary ganglion of the chick *Z Zellforsch* 1965 67 835-813
- LAFFEL A I, P I SAWANT and S SHIRKO Lysosomes: Distribution in animals, hydrolytic capacity and other properties. In *Ciba Foundation Symposium: Lysosomes* Churchill London 1963 78-113
- FAXI J La distribution des cholinestérases dans divers ganglions du système nerveux autonome des Vertébrés *Bibl anat (Basel)* 1961 2 73-89
- ГРОИДРОВ В On the histochemical localization of the non specific cholinesterase in the central nervous system of rats *Dokl Akad Nauk (Sofia)* 1962 15 443-446
- TEWARI H B and G H BOURNE Histochemical localization of specific and non specific cholinesterases and simple esterase in myelinated nerves *Exp Cell Res* 1960 21 245-248
- TEWARI H B and G H BOURNE Absence of specific cholinesterase from cerebellar synaptic functions and localization of other enzymes *Exp Cell Res* 1962 27 173-177
- TEWARI H B and G H BOURNE The histochemistry of the nucleus and nucleolus with reference to nucleo-cytoplasmic relations in the spinal ganglion neuron of the rat *Acta histochem (Jena)* 1962 a 13 322-350
- TEWARI H B and G H BOURNE Histochemical studies of the distribution of oxidative enzymes in the cerebellum of the rat *J Histochem Cytochem* 1962 b 10 619-627
- TEWARI H B and G H BOURNE Histochemical evidence of metabolic cycles in spinal ganglion cells of rat *J Histochem Cytochem* 1962 c 10 42-64
- TEWARI H B and G H BOURNE On the topographical differences in the localization of certain enzymes in trigeminal ganglion cells of rat *Experientia (Basel)* 1963 19 238-240
- TEWARI H B and G H BOURNE Histochemical studies on the distribution of adenosine triphosphatase in the trigeminal ganglion cells of the rat *J Histochem Cytochem* 1963 a 11 511-519
- TEWARI H B and G H BOURNE Histochemical studies on the localization of adenosine triphosphatase in the cerebellum of the rat *J Histochem Cytochem* 1963 b 11 246-257
- TEWARI H B and G H BOURNE On the histochemical demonstration of alkaline and acid phosphatase and simple esterase in the neurons and «boutons terminaux» of the cerebrum of the rat *J Histochem Cytochem* 1963 c 11 116-118
- TEWARI H B and G H BOURNE Histochemical studies on the distribution of monoamine oxidase in the cerebellum of rat *Acta anat (Basel)* 1963 d 57 334-340
- TEWARI H B and G H BOURNE Histochemical studies on the distribution of alkaline and acid phosphatases and nucleotidase in the trigeminal ganglion cells of rat *Acta histochem (Jena)* 1964 17 197-207
- THOMAS E Dehydrogenasen und Esterasen in unveränderten und geschädigten Spinalganglienzellen vom Menschen. Histochemische Untersuchung an einem cervicalen Ganglion mit einem angiomatösen Meningiom *Acta neuropath (Berl)* 1963 2 231-241
- THOMAS E and A G E PEARIE The fine localization of dehydrogenases in the nervous system *Histochemistry* 1961 2 266-282

- TORACK R M Adenosine triphosphatase activity in rat brain following differential fixation with formaldehyde glutaraldehyde and hydroxydipaldehyde *J Histochem Cytochem* 1965 13 191-203
- TORACK R M and R J BARNETT Fine structural localization of cholinesterase activity in the rat brain stem *Exp Neurol* 1962 6 224-244
- TORACK R M and R J BARNETT Nucleoside phosphatase activity in membranous fine structures of neurons and glia *J Histochem Cytochem* 1963 11 763-772
- TOICHI G A biochemical study of brain microsomes *Exp Cell Res* 1959 16 232-255
- TUM SUDEN C ■ R HART, R LINDENBERG and A S MARRAZZI Pharmacologic and anatomic indications of adrenergic neurons participating in synapses at parasympathetic ganglia (ciliary) *J Pharmacol exp Ther* 1951 103 364-365
- TUM SUDEN, C E R HART and A S MARRAZZI Influence of acetylcholine on synaptic transmission in a parasympathetic ganglion (ciliary) *Fed Proc* 1952 11 164
- UNDERHAY F F, The hydrolysis of indoxyl esters by human blood *Biochem J* 1957 66 383-390
- VESCO C and A GIUDITTA Tetrazolium reductase activity of the enzymatic systems of oxidation-reduced nicotinamide nucleotides in mammalian brain *Biochem Biophys Acta* (Amst) 1966 113 197-215
- WACHSTEIN M, M BRADSHAW and J M ORTIZ Histochemical demonstration of mitochondrial adenosine triphosphatase activity in tissue sections *J Histochem Cytochem* 1962 10 65-74
- WACHSTEIN M and E MEISEL, A comparative study of enzymatic staining reactions in the rat kidney with necrobiosis induced by ischemia and nephrotoxic agents (mercurhydrin and DL-serine) *J Histochem Cytochem* 1957 5 204-220
- WACHSTEIN M and E MEISEL Histochemistry of «thioacetic acid esterases» in relation to Duve's lysosomes *J Histochem Cytochem* 1960 8 317-318
- WALKER D G and A M SELIGMAN The use of formalin fixation in the cytochemical demonstration of succinic and DPN and TPN dependent dehydrogenases in mitochondria *J Cell Biol* 1963 16 455-469
- WALTER W Über den Nachweis der alkalischen und sauren Phosphatasen in sympathischen Grenzstrangganglien und ihre Beeinflussung durch Pendiomid *Acta histochem (Jena)* 1955 1 1-14
- WATTENBERG L W The effects of quinones on the activity of flavoprotein dehydrogenase systems as measured by tetrazolium salt reduction *Ann Histochem* 1961 6 303-310
- WATTENBERG L W and J I LEONO Effects of coenzyme Q<sub>10</sub> and menadione on succinic dehydrogenase activity as measured by tetrazolium salt reduction *J Histochem Cytochem* 1960 8 296-303
- WAWRZYNIAK M The activity of adenosine triphosphatase in motoneurons of the spinal cord of the guinea pig *Folia Histochem Cytochem* 1963 1 57-75
- WEINER N The distribution of monoamine oxidase and succinic oxidase in brain *J Neurochem* 1960 6 79-86
- WHITTAKER V P The isolation and characterization of acetylcholine-containing particles from brain *Biochem J* 1959 72 694-706
- WHITTAKER V P, I A MICHAELSON and R J A KIRKLAND The separation of synaptic vesicles from nerve ending particles («synaptosomes») *Biochem J* 1964 90 293-303
- WHITTAKER V P and M N SHERIDAN The morphology and acetylcholine content of isolated cerebral cortical synaptic vesicles *J Neurochem* 1965 12 363-372
- WHITTAKERIDGE D The transmission of impulses through the ciliary ganglion *J Physiol (Lond)* 1937 89 99-111
- WOHLRAB F Der histochemische Nachweis der Monoaminoxidase mit Thiazolyl substituiereten Tetrazolumsalzen in Gegenwart von Metallionen *Histochemie* 1961 2 163-173
- WOLFGANG F and A S ROSE The histochemical demonstration of dehydrogenases in neuroglia *Exp Cell Res* 1959 17 526-530
- ZIMMERMANN H and A G C IREARSE Limitations in the histochemical demonstration of pyridine nucleotide linked dehydrogenases («nonthin dehydrogenases») *J Histochem Cytochem* 1959 7 271-275







ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 70 SUPPLEMENTUM 287

FROM THE DEPARTMENT OF PHARMACOLOGY UNIVERSITY OF UPPSALA SWEDEN

PROPAGATION VELOCITY  
IN HUMAN MUSCLE FIBERS  
IN SITU

BY

ERIK STALBERG

UPPSALA 1966





ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 70 SUPPLEMENTUM 287

FROM THE DEPARTMENT OF PHARMACOLOGY UNIVERSITY OF UPPSALA SWEDEN

PROPAGATION VELOCITY  
IN HUMAN MUSCLE FIBERS  
IN SITU

BY

ERIK STALBERG

UPPSALA 1966

CHAPTER 4 <i>Propagation velocity during prolonged activity</i>	40
Summary	40
Introduction	40
Methods	40
Results	41
Continuous contraction 41 — Influence of the discharge frequency on the propagation velocity 42 — Effect of pauses 44 — Action potential amplitude 45	
Discussion	46
CHAPTER 5 <i>Propagation velocity over long distance</i>	51
Summary	51
Introduction	51
Methods	51
Electrical activation 51 — Voluntary activation 52	
Results	52
Electrical activation 52 — Voluntary activation 54	
Discussion	54
Direct muscle stimulation 54 — Electrical stimulation of intramuscular nerve twigs 54 — Voluntary activation 58	
CHAPTER 6 <i>Variability in propagation velocity for consecutive discharges in a fiber</i> *The Velocity Recovery Function	60
Summary	60
Introduction	60
Methods	60
Double pulse stimulation 60 — Stimulation with trains of pulses 61 — Stimulation with irregular stimulation rhythm 61 — Voluntary activation 61	
Results	62
Double pulse stimulation 62 — Trains of pulses 63 — Stimulation with irregular rhythm 64 — Voluntary activation 64	
Analysis of the Velocity Recovery Function (VRF)	68
Discussion	74
CHAPTER 7 <i>Propagation velocity during ischemia</i>	78
Summary	78
Introduction	78
Methods	78
Results	79
General findings 79 — Propagation velocity 80 — The Velocity Recovery Function 81 — Temperature 83	
Discussion	85

CHAPTER 8 <i>Propagation velocity at different temperatures</i>	89
Summary	89
Introduction	89
Methods	90
Results	90
General findings 90 — Propagation velocity 91 — The Velocity Recovery Function 92	
Discussion	94
CHAPTER 9 <i>Propagation velocity in different muscles</i>	96
Summary	96
Introduction	96
Methods	97
Results	97
Discussion	99
CHAPTER 10 <i>Some findings in muscular disorders</i>	101
Summary	101
Introduction	101
Methods	101
Results	101
Propagation velocity 101 — Velocity Recovery Function 102	
Discussion	102
General summary	104
Acknowledgements	107
References	108



## GENERAL INTRODUCTION

Electromyography (EMG) was introduced for clinical use by Piper in 1912. He used surface electrodes. A great improvement on the technique was made by Adrian & Bronk in 1929 who introduced the concentric needle electrode. The method has since been developed into a useful tool for the diagnosis and study of muscular diseases.

In ordinary electromyography the summated activity from a great number of muscle fibers is recorded. Fibers innervated from the same motoneuron and therefore activated simultaneously, generate the motor unit potential. The EMG gives information about the total electrical activity within a certain area in the vicinity of the electrode. The properties of the single muscle fiber are not investigated with this method.

Although the examination of single muscle fibers has long been desired there have been considerable difficulties in doing this as a clinical routine test. Glass capillary electrodes for measurements of the resting potential have been used by Johns (1960), Norms (1962), Creutzfeldt (1962) and Riecker, Bolte & von Bubnoff (1963). Beranek (1961), van der Most, van Spijk, den Hertog & Lammers (1965) and den Hertog (1966) and Goodgold & Eberstein (1966) recorded intracellular action potentials with a similar method.

Pierce & Wagman (1964) used a tungsten microelectrode for extracellular recording of action potentials in human muscle fibers *in situ*. The electrode (with a tip diameter of  $1 \mu$ ) was inserted percutaneously and held by a micromanipulator.

The needle Multielectrode method of extracellular recording of single muscle fibers was first reported by Ekstedt & Stalberg (1963). The method was described in detail by Ekstedt (1964). The method is based on the use of a needle Multielectrode and is simple and fast. During a one hour experimental period action potentials from 30 different fibers could be recorded. However, the knowledge of the volume conduction of the action currents of a muscle fiber is at present not such as to permit any conclusions concerning the events of the muscle fiber membrane itself to be drawn from the shape of the extracellularly recorded action potentials.

The needle Multielectrode technique also permits recording at two sites along the fiber and makes possible the calculation of the propagation velocity of the action potential along the muscle fiber in situ which has not previously been studied

The aim of the present paper is to develop a method for the study of propagation velocity in the normal muscle. The propagation velocity has also been studied in different muscles in muscular disorders and during prolonged activity ischemia and changed intramuscular temperature

The investigations showed that the propagation velocity was subject to continuous changes during the experiment related to activity. This made it difficult to use any instant numerical value of the propagation velocity as a parameter to characterize the fiber. However when recording for longer periods (more than 5 minutes) the change in propagation velocity might give some information. It was also found that there was a variability in the propagation velocity at consecutive discharges. This variability was related to the innervation frequency in such a way that a short time interval from the preceding impulse enhanced the velocity for the second pulse. This effect was the more pronounced the shorter the intervals between the discharges. The analysis of this interval dependence the *Velocity Recovery Function* has given results that seem promising with regard to the characterizing of the muscle fiber membrane properties. The Velocity Recovery Function changed during prolonged work during ischemia at low temperature and in some muscular disorders preliminarily studied.

The following preliminary reports of results presented in this paper have been given Ekstedt & Stålberg (1963) Stålberg & Ekstedt (1965) Stålberg Dahlbäck & Ekstedt (1966)

# SURVEY OF THE LITERATURE

## *Measurements of propagation velocity in skeletal muscle in situ*

The first investigation on propagation velocity in the skeletal muscle was performed on the human forearm with surface electrodes by Herman (1878) who found dass die muskuläre Leitungsgeschwindigkeit im menschlichen Vorderarm auf keinen Fall 16 Meter übersteigt und wahrscheinlich zwischen 10 und 13 Meter liegt. Due to the inaccurate methods used this investigation has but historical interest. Mainly the same method (one surface electrode below the elbow, one proximal to the wrist) was used by Pipier (1909) who found the impulse velocity in the forearm to be 10 m/sec. The localization of the motor end plates was not taken into account.

In 1939 Eccles & O'Connor when studying neuromuscular transmission, recorded electrical activity from the cat soleus, anterior tibial and peroneus tertius muscles by means of two stout collars of cotton wool around the muscle. The values ranged in the soleus muscle from 2.85 to 4.8 m/sec and in the peroneus tertius and anterior tibial muscles from 5 to 6 m/sec. Accurate measurements of the fastest conduction velocity could not be made but was assumed to be only slightly higher.

Denslow & Hassett (1943) made measurements of propagation velocity on motor unit potentials in the voluntarily activated human muscle by inserting two concentric needle electrodes in the fiber direction in different muscles. The 24 values of the propagation velocity were evenly spread between 1.3 m/sec and 12.5 m/sec with mean value of 4.95 m/sec. No consideration was taken of the site of the end plate in relation to the recording electrodes and therefore these results did not represent the velocity in the muscle fibers.

With two pairs of two 125  $\mu$  platinum wires spaced 3–10 mm Jarcho Berman, Dowben & Lilienthal (1954) measured the conduction velocity in denervated and normal muscle fibers in the anterior gracilis rat muscle. In normal fibers the velocity ranged from 3.56 to 5.63 m/sec (mean 4.3 m/sec) and in denervated fibers the velocity also fell within this range but fibrillary potentials in denervated fibers had a velocity of 2.66 to 2.84 m/sec (mean 2.73 m/sec).

The first measurements of propagation velocity by means of two electrodes



within one needle was published by Meda & Ferroni (1957). In the bevelled surface at the tip of a needle (diameter of 1.45 mm) two platinum electrodes, 60  $\mu$  in diameter were mounted 1.25 mm from each other. The needle was so positioned that the electrodes were grossly parallel to the fiber direction and the propagation time was measured on enlarged photos from the interval between the recorded potentials. In m. quadriceps femoris the mean velocity in 6 healthy subjects was found to be 4.26 m/sec SD 0.782  $n=134$ .

Buchthal, Guld & Rosenfalck (1955a, b) noticed that in the measurements of Denslow & Hassett (1943) it was impossible to ascertain whether the electrodes were positioned on the same fiber. They thus considered the results achieved not to reflect the true distribution of propagation velocity in the muscle. To overcome these difficulties Buchthal *et al.* used a method in which they stimulated a small group of fibers in the brachial biceps in man by an intramuscular needle electrode and picked up the evoked muscle potentials with 3-5 concentric electrodes at some centimeters distance from each other along the fiber direction. After 1-2 hours of searching it was possible to find a position of the needles where the recorded potentials appeared and disappeared simultaneously when the stimulation strength was changed and where there was a rectilinear relationship between the arrival time of the potentials recorded from different electrodes and the electrode distance. The propagation velocity in these measurements was found to be 4.02 m/sec SD 0.45  $n=7$ . In another series of experiments Buchthal, Guld & Rosenfalck (1955b) presented the same type of experiments with voluntarily activated muscles. The electrode positions were found with electrical stimulation as described above. There was a variation in velocity exceeding by far that obtained with electrical stimulation and the authors commented that "this difference must be due to spatial dispersion of the site of innervation for simultaneously activated fibers: the spikes recorded at the different electrodes not necessarily arising from the same fibers of the motor unit". The velocity was found to be 4.72 m/sec SD 0.54  $n=28$ . No difference in velocity was found between children, adults and aged persons. No measurements of propagation velocity during prolonged continuous activity were made.

In 1961 Sjöström used vector recordings from two electrodes spaced up to 40 mm. When a Lissajous figure appeared on the oscilloscope it could be concluded that the electrodes recorded activity from fibers belonging to the same motor unit but no attempt appears to have been made to ascertain that the recordings were made from a single fiber. Time was measured between the negative peaks of the potentials and the velocity was calculated to be 8.3, 7.1, 5.0 m/sec respectively (mean value 6.8 m/sec) in different parietic muscles.

Even if there are reports in the literature on measurements of propagation velocity in human muscle only a few of these might represent measurements from single fibers

### *Propagation velocity and fiber diameter*

Recent reviews of electrical properties in excitable tissue is given by Ussing (1959) and Noble (1966). Different models used for calculating the propagation velocity were discussed by Håkansson (1956).

Since the prediction of Gasser & Erlanger (1927) that there was a proportionality between the conduction velocity and fiber diameter in myelinated nerve many investigations have shown this to be true both for the myelinated and non myelinated nerve fibers. The results are however, very conflicting.

Since the velocity in the *myelinated nerve fiber* might be influenced by the myelin sheath and the inter nodal distance the relationship between velocity and fiber diameter is not analogous to that in the muscle fiber.

In *non myelinated nerve* (giant axon of the squid) Pumphrey & Young (1938) correlated the largest diameter in the fiber bundle and the shortest propagation time in that bundle and found the velocity to vary approximately with the square root of the fiber diameter. Offner, Weinberg & Young (1940) obtained the same results in their mathematical analysis while Gasser (1950) and Hodges (1953) found a linear proportionality in the non myelinated nerve of the cat.

Katz (1948) supposed the correlation to be essentially the same in *muscle fibers* as in non myelinated nerve fibers. Håkansson (1954, 1956) made the first systematic investigation of the relationship between propagation velocity and diameter of the isolated muscle fiber. Because the fibers of the semitendinosus muscle in the frog have an elliptic cross section the size of the fibers were characterized by their circumferences. It was found that the conduction velocity was directly proportional to the fiber circumference.

For the human brachial biceps muscle *in situ* Buchthal, Guld & Rosenfalck (1955b) found a narrow range of velocities in different muscle fibers as compared to the three fold variation in fiber diameter. They considered the inactive fibers as insulators and the extracellular space smaller than had earlier been reported (6 % instead of 13 %) which should give a high external resistance masking the influence of fiber diameter.

within one needle was published by Meda & Ferroni (1957). In the bevelled surface at the tip of a needle (diameter of 1.45 mm) two platinum electrodes, 60  $\mu$  in diameter were mounted 1.25 mm from each other. The needle was so positioned that the electrodes were grossly parallel to the fiber direction and the propagation time was measured on enlarged photos from the interval between the recorded potentials. In m quadriceps femoris the mean velocity in 6 healthy subjects was found to be 4.26 m/sec SD 0.782 n=134.

Buchthal, Guld & Rosenfalck (1955a, b) noticed that in the measurements of Denslow & Hassett (1943) it was impossible to ascertain whether the electrodes were positioned on the same fiber. They thus considered the results achieved not to reflect the true distribution of propagation velocity in the muscle. To overcome these difficulties Buchthal *et al.* used a method in which they stimulated a small group of fibers in the brachial biceps in man by an intramuscular needle electrode and picked up the evoked muscle potentials with 3-5 concentric electrodes at some centimeters distance from each other along the fiber direction. After 1-2 hours of searching it was possible to find a position of the needles where the recorded potentials appeared and disappeared simultaneously when the stimulation strength was changed and where there was a rectilinear relationship between the arrival time of the potentials recorded from different electrodes and the electrode distance. The propagation velocity in these measurements was found to be 4.02 m/sec SD 0.45 n=7. In another series of experiments Buchthal, Guld & Rosenfalck (1955b) presented the same type of experiments with voluntarily activated muscles. The electrode positions were found with electrical stimulation as described above. There was a variation in velocity exceeding by far that obtained with electrical stimulation and the authors commented that "this difference must be due to spatial dispersion of the site of innervation for simultaneously activated fibers: the spikes recorded at the different electrodes not necessarily arising from the same fibers of the motor unit". The velocity was found to be 4.72 m/sec SD 0.54 n=28. No difference in velocity was found between children, adults and aged persons. No measurements of propagation velocity during prolonged continuous activity were made.

In 1961 Seno used vector recordings from two electrodes spaced up to 40 mm. When a Lissajous figure appeared on the oscilloscope it could be concluded that the electrodes recorded activity from fibers belonging to the same motor unit but no attempt appears to have been made to ascertain that the recordings were made from a single fiber. Time was measured between the negative peaks of the potentials and the velocity was calculated to be 8.3, 7.1, 5.0 m/sec respectively (mean value 6.8 m/sec) in different paretic muscles.

Even if there are reports in the literature on the conduction velocity in human muscle, only a few of these have been derived from single fibers

## Propagation velocity and fiber diameter

Recent reviews of electrical properties in excitable tissues (1959) and Noble (1966) Different models used for conduction velocity were discussed by Håkansson (1956)

Since the prediction of Gasser & Erlanger (1927) of a proportionality between the conduction velocity and fiber diameter in myelinated nerve many investigations have shown this to be true for the myelinated and non myelinated nerve fibers. The results are very conflicting.

Since the velocity in the myelinated nerve fiber might be determined by the myelin sheath and the inter nodal distance the relation between velocity and fiber diameter is not analogous to that in the muscle fiber.

In non myelinated nerve (giant axon of the squid) Purshouse & Taylor (1938) correlated the largest diameter in the fiber bundle and the conduction propagation time in that bundle and found the velocity to vary approximately with the square root of the fiber diameter. Olfner, Weinberg & Yarnitzky (1940) obtained the same results in their mathematical analysis while Gasser (1950) and Hodges (1953) found a linear proportionality in the non myelinated nerve of the cat.

Katz (1948) supposed the correlation to be essentially the same in muscle fibers as in non myelinated nerve fibers. Håkansson (1954, 1956) made the first systematic investigation of the relationship between propagation velocity and diameter of the isolated muscle fiber. Because the fibers of the semitendinosus muscle in the frog have an elliptic cross section the size of the fibers were characterized by their circumferences. It was found that the conduction velocity was directly proportional to the fiber circumference.

For the human brachial biceps muscle *in situ* Buchthal, Guild & Rosenfalck (1955b) found a narrow range of velocities in different muscle fibers as compared to the three fold variation in fiber diameter. They considered the inactive fibers as insulators and the extracellular space smaller than had earlier been reported (6% instead of 13%) which should give a high external resistance masking the influence of fiber diameter.

## METHODS

*Recording***The electrode probes***Probes for measurement of the propagation velocity*

The method was based on the use of Multielectrode probes of the same type as described by Ekstedt in 1964

Fourteen platinum wires individually isolated were introduced in an ordinary injection cannula Two millimeters from the point opposite to the bevel surface they were bent up through an opening 0.5–1 mm in the cannula The wires were arranged in a certain geometrical pattern and fixed in epoxy resin (Araldite® Ciba) The needle and isolator were ground flat The diameters of the needle were 0.6 and 0.5 mm Each wire was connected to its pin in a multipolar plug As seen in Fig. 1 there were thirteen electrodes in one row and one single electrode 240  $\mu$  from the center of the row

The distance between electrode centers was about 30  $\mu$  and each electrode 25  $\mu$  in diameter

This Multielectrode with small interelectrode distances was the one most suitable for studies of propagation velocity In addition other types of multi-electrode probes were used for example the "3+11" and "3×4" Multielectrodes (Fig. 2) These were also used by Ekstedt (1964)

*The reference electrode*

In most of the experiments a concentric needle electrode subcutaneously inserted was used as reference electrode Before recording it was controlled that no electrical activity was picked up from this electrode Sometimes one of the outer electrodes in the multielectrode probe was used as reference for example when studying the cross talk (page 26)

**Electrical properties of the recording equipment**

A detailed description of the recording system and the method used for testing the properties is given by Ekstedt (1964) Here only some of the most important characteristics will be given

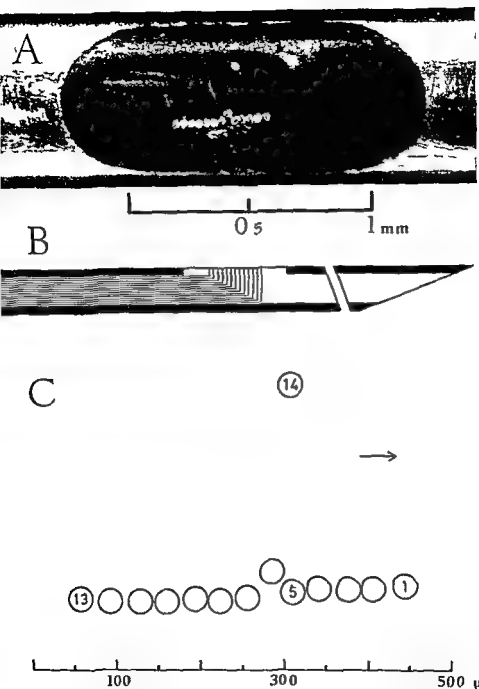


FIG 1 *The 1+13 Multielectrode* A Microphotograph of the electrode array B Schematic drawing of a longitudinal section of the needle showing the platinum wires embedded in epoxy resin and ending in the side of and flush with the needle C The electrode array redrawn from a microphotograph

A

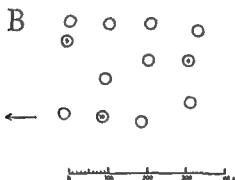
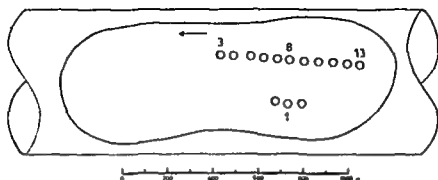


FIG 2 The electrode array of A The 3+11 Multielectrode and B The 3x4 Multielectrode. The arrows indicating the points of the needles

### *Electrical properties of the amplifiers without the electrode*

The input impedance was 250 Mohm and the input capacitance 3-5  $\mu\text{F}$

The frequency response curve was flat between 1 and 20 000 Hz and reduced 3 dB at 0.5 and 40 000 Hz. Phase distortion and nonlinear distortion was negligible

Common mode rejection was more than 700 times for any recording condition

### *Electrical properties of the amplifiers and the electrode connected*

The frequency response curve was flat between 2 and 20 000 Hz and decreased 3 dB at 1 and 35 000 Hz

Common mode rejection was more than 300 and usually 500 times

The 10-90 % rise time for the whole system was 12  $\mu\text{sec}$

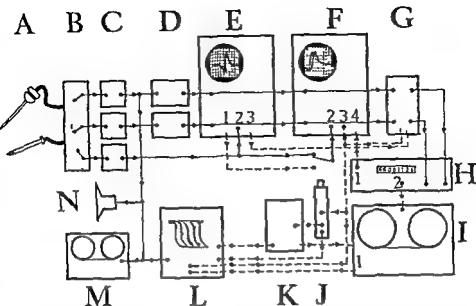


FIG 3 The experimental set up A The Multielectrode and the subcutaneous reference electrode B Input cathode follower and electrode selector Each electrode in the Multielectrode is connected to its own cathode follower and its connecting wire separately cathodally screened C The amplifiers with attenuators D The Delay Units in which the action potentials can be delayed up to 500  $\mu$ sec E Dual beam oscilloscope (E1=sweep sawtooth signal output E2=External trigger input E3=Gate signal output The gate signal is simultaneous with the oscilloscope sweep) F Dual beam oscilloscope which is photographed (F2=External trigger input F3=Gate signal output F4=input for intensity modulation of the oscilloscope beam) G The Gate Unit by which the action potentials are prevented to reach the Time Interval Counter except during the passage of a sweep on oscilloscope E or F H The Time Interval Counter (H1=output for marking pulses that gives brightened spots on the action potential on oscilloscope F coincident with the counter start and stop H2=the binary coded decimal output to the Magnetic Tape Unit) I The Magnetic Tape Unit (I1=event markings to the mk jet recorder) J Oscilloscope camera (governed by F) K Time marker giving synchronous signals to camera Magnetic Tape Unit and ink jet recorder L Ink jet recorder for action potentials and event markings M Tap recorder for study of the innervation frequency N Loudspeaker with its amplifier

### The Delay Unit

In order to be able to see the initial part of an action potential ahead of the triggering point a special device was constructed By means of this the action potential before it was displayed on the oscilloscope screen, could be delayed in relation to the original signal which triggered the sweep This Delay Unit (D in Fig 3) was built up around identical electromagnetic delay



lines in which the signal could be delayed from 0 to 500  $\mu$ sec in 10  $\mu$ sec steps (Czekajewski, Ekstedt & Stålberg to be published). The rise time 10-90 % was 3 % of the actual delay time i.e. 15  $\mu$ sec for 500  $\mu$ sec delay. High frequency response gradually decreased from 40,000 to 32,000 with increasing delay time from 0 to 500  $\mu$ sec. There was no phase distortion or nonlinearity within the passband. The Delay Unit had two channels that were identical in every respect.

### Action potential display

The potentials were displayed on two dual beam oscilloscopes (Tektronix type 502 and 502A) with P11 phosphor. The oscilloscopes were triggered by the potentials themselves e.g. by the initial positive phase. This part of the potential was rather slow and therefore interference from distant active fibers sometimes gave fluctuations of the trigger point on the potential from discharge to discharge and an unstable oscillographic picture. To get it more steady the triggering was sometimes made by the rising phase of the potential which was 4 times faster than the initial positive phase and therefore the trigger point was less sensitive to background activity. The optimal stability of the oscilloscope display was attained when the base line intersection of the potential was used for triggering.

The triggering technique normally used means that only the largest potentials can be dissected free of a number of nearly simultaneously appearing signals. The problem of triggering action potentials at option even small ones was not easily solved except in one special case namely when the small potentials came in a fixed time relation to a large triggerable signal. At voluntary contraction this occurs when two action potentials belonging to the same motor unit could be recorded at the same time. To get only the small signal displayed the triggering was made in the following way. One oscilloscope was triggered in the ordinary way by the large first action potential and with a sweep velocity, permitting both potentials to be seen. The second oscilloscope was then triggered upon the horizontal deflection voltage from the first oscilloscope and thereby an optional delay of the trigger of the second oscilloscope could be obtained.

One of the oscilloscopes was photographed by a 16 mm movie camera (Beaulieu R16). By a special device the camera shutter was open during the sweep after which a new frame was advanced. The camera followed the oscilloscope up to a maximal frequency of 15/sec. Several action potentials could also be superimposed on one frame. Exposure number and time was indicated on each frame.

## Ink jet recorder

For simplifying the analysis of the experiments a three channel ink jet recorder (modified Mingograph type 24 B) was used. On this the total activity from the electrode was recorded as well as time markings and event markings for oscilloscope sweeps for recordings on the digital tape and for moments of photographing with the oscilloscope camera.

### Measurements of propagation time

During the development of the method the propagation time i.e. the time for an action potential to travel the distance between two electrodes, was determined by measuring the distance between the base line intersection points of the two action potentials on an enlarged photograph.

Later the method of "sweep wobbling" was developed to increase the accuracy of the measurements and to facilitate the control of the needle position. The horizontal deflection voltage of the oscilloscope was modulated

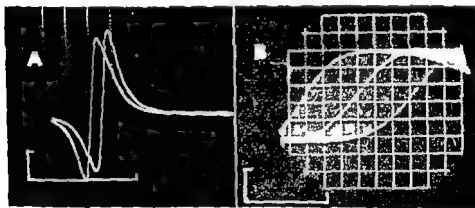


FIG. 4 Action potentials from electrodes 1 and 8 in the 3+11 Multielectrode. A Calibration 2 mV and 500  $\mu$ sec. The propagation time is measured between the baseline intersection points of the two action potentials. Upward deflection means negative voltage. Note that the two action potentials are of somewhat different form: the negative peak of the first potential and the positive peak of the second potential are slightly rounded due to cross talk between the electrodes (cf. page 26). The total amplitude is the same. B Calibration 2 mV 100  $\mu$ sec. The sweep wobbling method of measuring the propagation velocity used at an early stage of the investigation (in Chapter 9). The sweep voltage is modulated by a 1 Mc sine wave which makes the two action potentials appear as broad bands. The voltage of the 1 Mc signal that is needed to make the two bands just touch each other in the rising phases of the action potentials is proportional to the propagation time.

by a 1 Mc AC signal. The spot then wobbled to and fro one million times a second and the action potentials appeared as broad bands (Fig. 4) the width of which was proportional to the voltage of the modulating signal. The wobbler voltage necessary to make the two bands just touch each other in the region of the steep rising phases was a measure of the time difference between the action potentials. By means of known time intervals a calibration curve was made for the conversion of potentiometer values to actual time.

The wobbler voltage was adjusted with a 10 turn potentiometer and the readings were made from its scale and were dictated on a tape recorder and usually also registered on a potentiometer recorder (Varian G 10). The time intervals for 5-10 discharges were constant within 5  $\mu$ sec. With the use of an oscilloscope sweep of 20  $\mu$ sec/cm the maximum error of the method was  $\pm 5 \mu$ sec.

In the last 300 experiments time intervals were measured with a Time Interval Counter consisting of an Electronic Counter (Hewlett Packard 5245L) with Time Interval plug in unit (Hewlett Packard 5262A). The maximal error was  $\pm 0.1 \mu$ sec ("last digit error"). The drift was less than three parts in  $10^4$  per day. It was important to start and stop the counter from corresponding points on the action potentials so chosen that the time interval should be insensitive to small variations in the potential amplitude. The base line intersection was the most stable and reliable point for this purpose. However noise and action potentials from other motor units also passed the base line and triggered the counter but this was prevented by a special gating device by which only those signals that were displayed on one of the oscilloscopes were permitted to reach the counter (H in Fig. 3). The moments for start and stop were indicated by bright spots on the action potentials on the oscilloscope.

The digital read out tubes of the counter were photographed by a camera (Bealieu R 16) which followed the counter to a maximal frequency of 15/sec. The data from the film was punched on cards for calculations on a digital computer.

In the last 100 experiments the time interval readings from the counter were directly recorded in IBM computer compatible code on magnetic tape (Magnetic Tape Unit model DY 2546 Dymec). Besides the propagation time the actual time from the beginning of the experiments in milliseconds was recorded for each impulse as well as the interelectrode distance used and the markings for different events during the experiments. The maximal average recording rate was 19 data per second asynchronously. However by means of a memory in which the information was stored before it was written on the tape the shortest interval between occasional measurements was 2 msec. The tape was directly used as an input medium to the computer.

## Measurements of interspike interval

The time interval between the consecutive discharges were originally in directly determined from the recordings on the ink jet recorder with a paper speed of 100 mm/sec with an approximate accuracy of 5 msec. Later the measurements were made in the following way: impulses from the oscilloscope ("the gate pulses"), simultaneous with the sweeps that were triggered by the potentials, were recorded on a tape recorder (Ampex type 122) with a tape speed of 19 cm/sec. These pulses were then played back into the Time Interval Counter and the distance between each measured. The maximal error was 0.1 msec. When using the digital Magnetic Tape Unit the time intervals for each impulse were automatically measured by the Time Interval Counter and recorded on the magnetic tape. The maximal error was  $\pm 1$  msec ("last digit error").

## Stimulation

For electrical stimulation a double pulse generator was used with variable pulse width, frequency, voltage and intervals between pulse pairs. A doubly shielded output transformer was used where the secondary shield was isolated from ground and connected to the shaft of the stimulation needle. (Buchthal, Guld & Rosenfalck, 1955a)

A bipolar stimulation needle (DISA 13k14) was used. In order to simulate a physiological uneven innervation rhythm the stimulator could be triggered by tape recorded pulses i.e. the gate pulses from an oscilloscope triggered by voluntary action potentials.

## Temperature measurements

In most of the experiments temperature was measured with a thermoprobe consisting of a copper-constantan thermocouple close to the tip within a 0.6 mm injection cannula.

The reference junction was kept in an oil bath of temperature  $35.0^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ . The thermal EMF was measured on a DC microvoltmeter (Philips GM 6020). The SD of single readings was about  $0.1^{\circ}\text{C}$ . The systematic error in temperature measurements due to thermal conduction in the thermoneedle and the recording Multielectrode (Buchthal, Hønske & Lindhard, 1944) is not known. As the two needles are of the same material and diameter and with the same length within the muscle the systematic change of temperature around the two needles should be of the same order of magnitude. The

by a 1 Mc AC signal. The spot then wobbled to and fro one million times a second and the action potentials appeared as broad bands (Fig. 4) the width of which was proportional to the voltage of the modulating signal. The wobbler voltage necessary to make the two bands just touch each other in the region of the steep rising phases was a measure of the time difference between the action potentials. By means of known time intervals a calibration curve was made for the conversion of potentiometer values to actual time.

The wobbler voltage was adjusted with a 10 turn potentiometer and the readings were made from its scale and were dictated on a tape recorder and usually also registered on a potentiometer recorder (Varian G 10). The time intervals for 5-10 discharges were constant within 5  $\mu$ sec. With the use of an oscilloscope sweep of 20  $\mu$ sec/cm the maximum error of the method was  $\pm 5 \mu$ sec.

In the last 300 experiments time intervals were measured with a Time Interval Counter consisting of an Electronic Counter (Hewlett Packard 5245L) with Time Interval plug in unit (Hewlett Packard 5262A). The maximal error was  $\pm 0.1 \mu$ sec ("last digit error"). The drift was less than three parts in  $10^6$  per day. It was important to start and stop the counter from corresponding points on the action potentials so chosen that the time interval should be insensitive to small variations in the potential amplitude. The base line intersection was the most stable and reliable point for this purpose. However noise and action potentials from other motor units also passed the base line and triggered the counter but this was prevented by a special gating device by which only those signals that were displayed on one of the oscilloscopes were permitted to reach the counter (H in Fig. 3). The moments for start and stop were indicated by bright spots on the action potentials on the oscilloscope.

The digital read out tubes of the counter were photographed by a camera (Bealieu R 16) which followed the counter to a maximal frequency of 15/sec. The data from the film was punched on cards for calculations on a digital computer.

In the last 100 experiments the time interval readings from the counter were directly recorded, in IBM computer compatible code on magnetic tape (Magnetic Tape Unit model DY 2546, Dymec). Besides the propagation time the actual time from the beginning of the experiments in milliseconds was recorded for each impulse as well as the interelectrode distance used and the markings for different events during the experiments. The maximal average recording rate was 19 data per second asynchronously. However by means of a memory in which the information was stored before it was written on the tape the shortest interval between occasional measurements was 2 msec. The tape was directly used as an input medium to the computer.

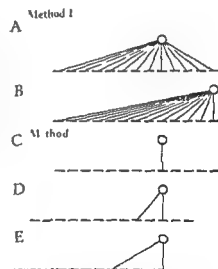
The subjects were ordered to keep up a slight constant contraction. Single muscle fiber potentials were usually easy to find and within 30–60 seconds from the insertion of the needle the recordings could begin.

During the experiment continuous small corrections of the needle position had to be made to keep the correct position. Recording of data was made only when optimal conditions were present. Ten to twenty potentials every 10 seconds were usually recorded but in the experiments where the digital type recorder was used thousands of consecutive action potentials were often recorded.

### *Recording of voltage decrement curves*

The decrease in action potential amplitude at increasing distance between the muscle fiber and the recording electrode here called the voltage decrement curves were usually obtained in the following way: the needle was positioned and the electrodes connected in the same way as described for recording the propagation velocity. The action potentials from electrode 14 were kept

FIG. 5. The two methods used to record action potentials at different distances from the same fiber (voltage decrement curves). The 14-13 Multielectrode is used. *Method 1*: The Multielectrode is kept in a fixed position in relation to the fiber and recording is made from all the electrodes one by one. In A the fiber overlies electrode 5; in B the fiber overlies one of the outer electrodes (number 1). *Method 2*: Recording is always made from electrode number 5 and the Multielectrode is for each recording moved so that the different electrodes one by one underlie the fiber. In C the fiber is positioned over electrode 5; in D and E the fiber is positioned over electrode 3 and 1 respectively and recording is made from electrode 5.



constant on one channel to provide a check of the needle position while potentials from each of the other electrodes were photographed (Figs. 5A and 6). In some experiments the needle was placed so that the fiber passed one of the outer electrodes (e.g. electrode 1) which also served as control of position. The other electrodes were then connected successively. In this way longer electrode-fiber distances were obtained (Fig. 5B and 7).

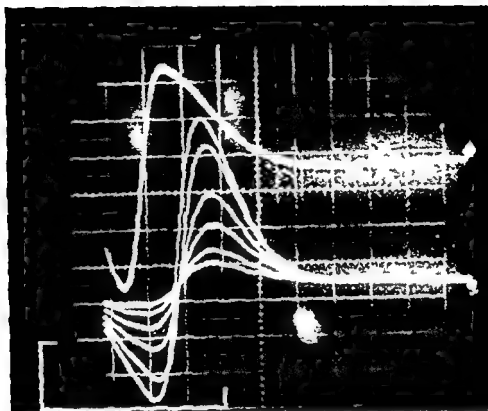


FIG 6 Voltage decrement recordings made according to method 1 in fig 5 Channel 1 6 superimposed action potentials from electrode 14 Channel 2 action potentials from electrodes 6-1

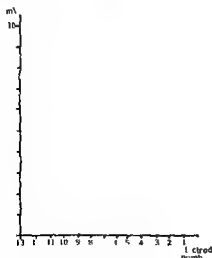
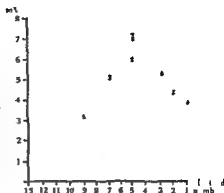


FIG 7 Amplitude of action potentials recorded at different distances from the fiber in one experiment Recording has been made according to II in fig 5A i.e. the fiber overlies electrode 1 and the recording is made from the electrodes in the long row in succession Note that the distances on the abscissa are the distances along the electrode row not the actual distances to the muscle fiber which are unknown Abscissa recording electrode The distance between electrode 13 (from which no recording was made in this experiment) and electrode 1 is 387  $\mu$  Ordinate Action potential amplitude in mV

FIG 11 Amplitude of action potentials recorded at different distances from the fiber (●) are the values obtained when recording is made according to A in fig 5 The fiber overlies electrode 4 and recording is made from the other electrodes in the long row The other values are obtained when recording was made according to C-E in fig 5 i.e recording was made from electrode 5 (+) and 14 (▲) while the Multi electrode was moved so that the electrodes underlay the fiber one by one



Another method for obtaining the voltage decrement curves was to move the Multielectrode so that the fiber passed over each electrode surface (Fig 5C-E) Recording was made from electrode 14 and from the corresponding electrode in the long row each time the fiber passed a new electrode (Fig 8)

### Analysis of data

Most of the calculations in this investigation have been made on the Control Data Corporation 3600 computer of the Uppsala Data Center The principles for the statistical analysis were taken from Snedecor (1956) (a test of differences p 45 analysis of regression and correlation p 122 curvilinear regression p 447) For calculating the mean propagation velocity the arithmetic mean has always been used

#### Addresses

Amper Redwood City California  
 Beaulieu Paris France  
 Ciba Ltd Basel Switzerland  
 Disa Elektronik Hørsholm Denmark  
 Dymec Division Hewlett Packard Co Palo Alto California  
 Elema Stockholm Sweden  
 Hewlett Packard Co Palo Alto California  
 Tektronix Beaverton Oregon USA  
 Philips Eindhoven Holland  
 Varian Ass Palo Alto California



## CHAPTER 2

# HOW TO POSITION THE MULTIELECTRODE CORRECTLY AND ESTIMATION OF ERRORS IN MEASUREMENTS

### SUMMARY

The following criteria for correct position of the Multielectrode for measurements of the propagation velocity were found

1 The action potentials from the two recording electrodes had maximum amplitude and parallel rising phases at the same position of the Multielectrode during to and fro movements

2 The measurement of the time interval should be made between the zero intersection points of the action potentials in the needle position when they were of maximum amplitude

3 During these recording conditions the maximal errors in measurement of propagation velocity over the needle was  $\pm 4\%$  to  $-2\%$

### INTRODUCTION

The Multielectrode method of determining the propagation velocity is based on measurements of small time intervals and distances and the results therefore are dependent upon the accurate positioning of the electrodes in relation to the muscle fiber

The present chapter is aimed at developing the criteria for correct needle position and to elucidate the sources of error in the method that are due to solely technical factors. The possible influence of the presence of the needle electrode upon the propagation velocity itself is discussed in Chapter 4

### *Procedures for placing the needle in correct position*

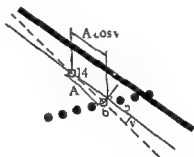
In order to be able to measure the propagation velocity of an action potential over a short distance (about 250  $\mu$ ) the exact position of the electrode in relation to the fiber has to be known for calculating the actual propagation length along the fiber. To get the exact propagation length the line between the two recording electrodes must be parallel with the fiber. When this is the case the distance between the electrodes is the same as the propagation length

## The "to-and fro" procedure

The action potentials from electrode 14 were displayed on one channel and the 1 + 13 Multielectrode was moved into the muscle until a fiber action potential was obtained. Then the electrodes in the long row were connected to the oscilloscope one by one, and the one giving the biggest action potential was chosen for recording and displayed on channel two.

The Multielectrode was now slightly moved to and fro ( $\pm 60 \mu$ ) in the direction of the needle axis. When the needle was out of the optimal position the amplitude was decreased, but the action potentials from the two electrodes closely resembled each other in form in all needle positions. When the fiber passed exactly over the connected electrodes, they registered maximal amplitude at the same position of the needle when it was moved to and fro.

FIG. 9. Wrong angle between the muscle fiber and the electrode row. The fiber passes electrode 14 to a point midway between electrodes 5 and 6. The propagation time measured between electrodes 14 and 6 will decrease with increase of the angle  $\gamma$ . All action potentials that are recorded from a line perpendicular to the fiber must have simultaneous base line intersections (see fig. 6). Therefore the length over which the propagation time is recorded must in this case be  $A \cos \gamma$  and the real distance between electrodes 6 and 14 is  $A$ . If the angle  $\gamma$  were  $90^\circ$  the propagation length should be zero and thus the base line intersections of the action potentials from electrodes 14 and 6 should be simultaneous.



## Experiments with wrongly positioned needle

Some experiments were performed to test the least error in needle position detected with the to and fro procedure.

When the electrode nearest to the optimal one was connected, it was seen that the recordings were of maximal amplitude at different positions of the needle. When the fiber was made to pass from electrode 14 to the area between two electrodes in the long row (the amplitude of the bipolarly recorded action potentials between these two electrodes was nearly zero) and the to and fro movements were made, it was just possible to detect the differences in needle position for maximal amplitude of the two potentials.

## CHAPTER III

# HOW TO POSITION THE MULTIELECTRODE CORRECTLY AND ESTIMATION OF ERRORS IN MEASUREMENTS

### SUMMARY

The following criteria for correct position of the Multielectrode of the propagation velocity were found

- 1 The action potentials from the two recording electrodes 1 and 2 should be parallel rising phases at the same position of the Multielectrode movements

- 2 The measurement of the time interval should be made at the same section points of the action potentials in the needle point of maximum amplitude

- 3 During these recording conditions the maximal errors in the propagation velocity over the needle was  $+4\%$  to  $-2\%$

### INTRODUCTION

The Multielectrode method of determining the propagation velocity is based on measurements of small time intervals and distances, and therefore are dependent upon the accurate positioning of the needle in relation to the muscle fiber

The present chapter is aimed at developing the criteria for correct position and to elucidate the sources of error in the measurements, and solely technical factors. The possible influence of the position of the electrode upon the propagation velocity itself is discussed in the next chapter.

### *Procedures for placing the needle in correct position*

In order to be able to measure the propagation velocity of the action potential over a short distance (about 250  $\mu$ ) the exact position of the electrode in relation to the fiber has to be known for calculation of the propagation length along the fiber. To get the exact position of the line between the two recording electrodes must be parallel to the fiber. When this is the case, the distance between the electrodes is equal to the propagation length.

When a fiber passed between electrodes 14 and 5, the cross talk of the action potentials in electrode 1 mounted by about 4 % and the volume conducted action potential by 10 % of the maximal amplitude. When switching from the subcutaneous electrode to electrode 1 as reference electrode the difference in the potentials from electrodes 14 and 5 (the fiber passing these electrodes) became less pronounced.

These experiments demonstrated that the electrodes were at the same distance from the fiber when the peak to peak amplitudes of the potentials were equal even if the ratio between negative and positive phase was unequal.

### *Experiments with rotation of the needle*

Now when the appearance of the recording for optimal position was known, some experiments were made to investigate the minimal error in needle position detected by means of this criterion.

The Multielectrode was rotated in both directions from the optimal position. Differences in the amplitude were obvious when the needle was rotated more than 20°. In this case the small potential had an amplitude of about 80 % of the large.

When the needle was incorrectly placed there was also a change in the steepness of the rising phase depending on a decreased amplitude and an increased rise time on one of the action potentials. For accurate study when the rising phases were non parallel certain corresponding points of the rising phases of the two potentials were superimposed by means of the Delay Unit. When the potentials were parallel all points of their rising phases coincided. The rising phases were seen to be non parallel when the needle was rotated about 10° and the differences in amplitude about 10 % with a sweep of 100  $\mu\text{sec/cm}$ . The change in propagation time could not be certainly determined in experiments but seemed not to exceed 2 %.

## Discussion

Cross talk was always seen to be more or less dependent on the amplitude and steepness of the rising phases of the action potentials. The cross talked signal was composed of two parts: one greater caused by the action potentials in the long row and one smaller caused by the action potential from electrode 14. Thus the recording from one electrode in the long row was influenced by an in phase cross talk from several electrodes and an out of phase cross talk from one electrode (number 14). The recording from electrode 14 was influenced only by out of phase cross talk from several electrodes. Because of the time interval between the potentials with the steepest part of the rising

phases approximately simultaneous with the positive or negative peak of the other potential the cross talk mainly influenced the amplitude of the potentials. If the electrodes had been more spaced causing a longer time interval between the recorded potentials, the cross talk would have influenced the slow positive or negative phases of the potentials (which was seen in the recordings with the  $3 \times 4$  Multielectrode).

In the  $1 + 13$  Multielectrode used in the measurements of propagation velocity even the electrodes in the end of the long row 1 and 13 recorded too large a volume conducted potential to be useful as reference. Therefore small differences in the potential forms had to be accepted. As the cross talk nearly always had its maximal influence before the positive and after the negative peak respectively the rising phases were not appreciably influenced. Therefore, even if one of the potentials was more distorted by cross talk than the other and their amplitudes therefore somewhat different the rising phases should be parallel in the correct needle position.

When the needle was rotated more than  $10^\circ$  out of correct position this was observed and corrected.

### *Reference point for time measurements*

Which part of the potential should be chosen for measurement of the time interval between the action potentials recorded from electrode 14 and one electrode in the long row?

When the potentials were identical in shape (except for the slight deformation caused by cross talk) and parallel in their rising phases it naturally did not matter which part of the potential was chosen for measurements as long as the measurements were made at the same level above or below the base line. If the measurements were made at different levels of the two potentials quite erroneous results were of course obtained. Therefore in all the experiments the measurements were made at the same level of the potentials within  $\pm 0.15$  mV which is  $\pm 1\%$  of maximal amplitude.

If however the potentials were not parallel because of small differences in the recording distances the measurements were influenced by the choice of reference point.

### *Experiments*

Some experiments were performed to evaluate the most stable and reliable reference point.

When the fiber passed 14-5 and recordings were made from the electrodes

in the long row one after the other it was at once seen that they had a common base line intersection Fig 6

When time measurements were made exactly on the base line about the same propagation time was obtained in the different recordings, except for the differences arising because the electrodes did not lie exactly on a straight line When the measurements were made above and below the base line shorter or longer time intervals were recorded Fig 11

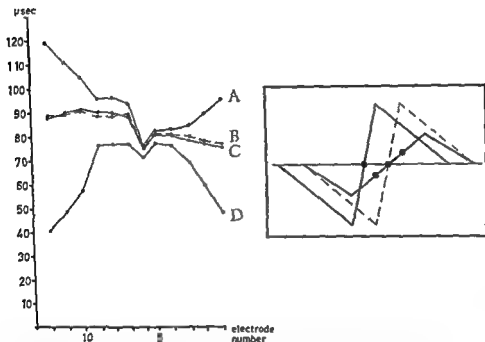


FIG 11 Influence of the reference point on the measured value of the propagation time The inset illustrates the principles the fiber is made to pass electrodes 14 and 5 in the 1+13 Multielectrode Measurement is first made between the base line intersections of the action potentials from these two electrodes then between electrodes 14-6 14-7 and so on The propagation times (ordinate) so obtained are plotted against the distance along the needle (abscissa) (curves B and C) The dip in the curves is caused by the fact that electrode 6 is positioned somewhat out of line The incline of the curve is caused by the fact that the long row is not strictly perpendicular to the line that joins electrodes 14 and 6 When measuring to a point 0.5 mV above the baseline of the second action potential (curve A) the time interval measured between electrodes 14 and 6 increases and this effect becomes considerably more pronounced when recordings from electrode 14 to one of the outer electrodes in the long row are made Similarly when increasing to a point 0.5 mV below the base line of the second action potential (curve D) the measured propagation time is decreased and this effect becomes more pronounced on the outer electrodes Thus the influence of incorrect position of the needle on propagation time must be least when measurement is made exactly in the base line intersection points of the two action potentials

When the measurements were made 1 mV from the base line on an action potential and picked up by an electrode nearest to the optimal one propagation time was increased or decreased by about 5 %

## Discussion

According to Lorente de No (1947) and Håkansson (1957) the base line intersection point of the action potentials corresponds to that moment when the depolarisation front is projected on the recording electrode perpendicular from the fiber. When therefore the action potentials were recorded at different distances from the fiber along the same line perpendicular from the fiber they had common base line intersections but different amplitudes and steepness of the rising phases. What is the magnitude of error introduced into time measurements due to inaccuracy in reference point? From the experiments with different reference points for measurements it could be calculated that when the fiber passed midway between two electrodes in the long row i.e. the position was just as inaccurate as to go undetected by the to and fro test and one reference point was 0.15 mV above and the other 0.15 mV below the base line the error in time measurements should be less than 0.2 %

### *The calculation of maximal errors of propagation time measurements because of wrong needle position with the recording still fulfilling the criteria for correct position*

The calculations will be based upon the fact known earlier and also confirmed in these investigations that the potential base line intersection is simultaneous for all action potentials recorded along a line perpendicular to the fiber.

The experiments demonstrated that when the two basic procedures for correct positioning have been carried out the velocity could still be erroneous due to some insensitivity in these procedures.

With the needle correctly positioned according to the to and fro procedure the fiber might nevertheless pass from electrode 14 to a point midway between two electrodes in the long row corresponding to a misalignment of 3.6 %. The decrease in actual propagation length can be trigonometrically calculated. The length will be  $A \cos v$  where  $A$  is interelectrode distance used in the calculations and  $v$  the angle between supposed and actual fiber direction (Fig. 9). The calculated velocity was  $A/t$  and the actual velocity  $A \cos v/t$ . For  $v = 3.6^\circ$  the velocity value thus should increase by 0.2 %

The same discussion was valid for the rotation experiments. When the needle was rotated  $10^\circ$  (which is the maximal error that passed undetected page 27) the actual distance was  $A \cos v$  and the velocity should be 1.5 % too high. Fig. 10

### *Alteration of the time interval due to distortion of the potential form in the recording system*

Some experiments were performed to obtain an expression of the maximal error caused by cross talk.

In the I+13 Multielectrode the outer electrodes usually registered a small volume conducted potential (5–10 % of maximal amplitude – see Fig. 6) and the cross talked signal. Time measurements were made when the signal from electrode 1 either a) was subtracted from the preceding potential b) added to the second potential or c) these combined.

This was made first when the propagation direction was 14–5 then when it was 5–14. Different results were obtained when an in phase signal or out-of phase signal was subtracted from the first or added to the second signal. The cross talk was mainly "in phase" with the recording obtained from the electrode in the row.

With this "artificial cross talk" the measured time could be changed by 3  $\mu\text{sec}$  shorter or longer. It seems reasonable to assume that during ordinary conditions the error was less than 1.5  $\mu\text{sec}$  and the variability of the error from discharge to discharge must be unimportant.

### *Inaccuracy of the time measurement equipment*

Most of the time measurements were made with the Time Interval Counter. The inaccuracy in time measurements was tested by measuring the time interval between one recorded action potential and the same action potential delayed 50  $\mu\text{sec}$  (with the Delay Unit, page 15). The time values obtained were 49.9, 50.0 and 50.1  $\mu\text{sec}$  ( $SD=0.038$ ,  $n=181$ ). The same results were obtained irrespective of whether a low, high or irregular discharge frequency was used. The test was also performed in the same way with sine waves with the same result.

### *Inaccuracy of measurement of interelectrode distance*

Microphotographs of the needle and an object micrometer scale (J.D. Møller Wedel in Holstein) were enlarged 2,000 times for measurements. The



accuracy was about  $\pm 2 \mu$  corresponding to  $\pm 4$  mm on the enlarged photograph

### *Evaluation of the errors tested with the 3×4 Multielectrode*

A single fiber potential was recorded over electrode 2 and 10. Then measurements were made 2-10, 2-6 and 6-10. The needle was then moved until the fiber passed 3-11 and new measurements were made in the same way. By moving the needle along its axis twelve different interelectrode distances 50, 80, 93, 103, 106, 144, 163, 184, 194, 244, 247 and 256  $\mu$  were obtained for measurements of propagation velocity. Representative results of one of the ten experiments are shown in Fig. 12. The experiments were made under especially unfavourable experimental conditions because large movements of the needle were necessary. The velocity decrease during these experiments seemed to be the same as in the normal experiments. The standard deviation of the velocity values (from the different distances) about the sliding mean was 0.15 m/sec with a range of 0.4 m/sec. In the experiment presented in Fig. 12 the recordings from the electrode pair 6-10 gave a lower

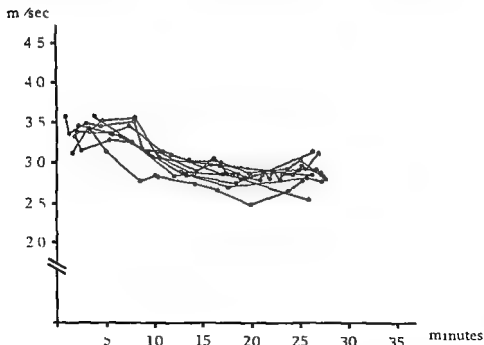


FIG. 12 Propagation velocity from one fiber recorded with the different electrode combinations in the 3×4 Multielectrode. Insertion of the Multielectrode at time zero

velocity but the measurements for the other short interelectrode distances did not seem to deviate systematically from the mean. It was somewhat difficult to get acceptable recordings when the shortest distance was used (1-5) as the cross talk seemed to influence the steep rising phase.

## Discussion

The systematic error arising from wrongly measured electrode distance and the effect of cross talk ought to cause measurements from certain electrode pairs to deviate significantly from the mean.

As shown earlier in this chapter the cross talk had negligible influence upon the measurements when the time interval between the potentials was more than about 50  $\mu$ sec (in these recordings valid for all measuring distances except for 1-5, 6-10 and 4-8) but when the interval was less the cross talk signal itself decreased in amplitude. The lower velocities obtained from the 6-10 distance cannot therefore reasonably be ascribed to the cross talk distortion but rather to unknown factors in this experiment. In other experiments the velocity measured over 6-10 did not differ from that over other electrode pairs.

The frequent repositions of the Multielectrode did not seem to influence the slope of the velocity time curve.

The variations in propagation time measurements were about 10 % (SD) in most of the experiments with the  $3 \times 4$  Multielectrode which is more than the errors in measurements with the  $1 + 13$  Multielectrode. This was partly due to difficulties in exactly positioning the  $3 \times 4$  Multielectrode because the electrodes were more spaced; with the to and fro procedure it was not possible to detect small misalignments in electrode position. If the needle was wrongly positioned in the plane of the electrodes and the electrode then was rotated to fulfill the amplitude and parallel criteria, two misalignments were superimposed and the errors in measurements added.

## CONCLUSIONS

The errors in measurements are of three different types:

1. Systematic errors constant through the investigation, mainly due to errors in measurements of the interelectrode distance which give a maximum error in propagation velocity of  $\pm 1$  %.
2. Errors due to technical factors that could change during the experiment and give slow variations of propagation velocity:
  - a) faulty positioning of the electrode which could give about 1.5 % too short a propagation time i.e. 1.5 % too high a velocity value.

- b) wrong reference point for time measurements, giving an increase or decrease in propagation value by 0.2 %
- c) error in the time interval between the potentials due to cross talk maximally  $\pm 1.5 \mu\text{sec}$  which corresponds to  $\pm 1 \%$  error in the propagation value if the correct propagation time is assumed to be  $75 \mu\text{sec}$

Should these factors (2a, 2b, 2c) all tend in the same direction the maximal error in propagation velocity should be +2.7 % to -0.2 %

3 Errors in measurements of consecutive action potentials (mainly due to last digit error in the Time Interval Counter) This was maximally 0.1  $\mu\text{sec}$  corresponding to 0.13 % for a propagation time of  $75 \mu\text{sec}$

Transient fluctuations in the mains voltage (stopping and starting machines in the building) could incidentally give erroneous values of the propagation time ( $\pm 5-10 \%$ ) during approximately one second. These errors were usually recognized but small changes might have passed undetected. The fault has been corrected and was not present during the last 27 experiments.

The maximal error in the measurements of propagation velocity are thus +4 % to -2 %

## STIMULATION OF MUSCLE FIBERS

## SUMMARY

1 For the stimulation in the human muscle *in situ* a bipolar stimulation needle electrode was used. For stimulation of the exposed rabbit muscle two tungsten wires were also used.

2 The most common type of response obtained was a polyphasic potential. Some times clean single fiber action potentials were obtained.

3 This type of response disappeared at curarisation of the rabbit. Therefore the muscle fibers were assumed to be activated indirectly via the nerve.

4 With the use of longer pulses and higher stimulation voltage coarse potentials of long duration without any spike components were obtained.

5 This type of response remained unchanged during curarisation of the rabbit and was therefore regarded as caused by direct stimulation of the muscle fibers. However it was not possible to limit the stimulation to one or a few muscle fibers.

## INTRODUCTION

This chapter is aimed at elucidating whether it is possible to obtain a single muscle fiber activity by direct electrical stimulation of the muscle *in situ*.

## METHODS

*Human experiments*

The experiments were performed on twelve subjects 22-35 years old and without signs of neuromuscular disease. The following muscles were used: the brachial biceps muscle (about 25 fibers in 5 different subjects), the extensor digitorum communis muscle (about 100 fibers in 12 different subjects).

Two electrode probes were used: one Multielectrode (I+13) and one bipolar stimulation electrode (DISA 13K14).

The stimulating electrode was positioned distally to the end plate zone, avoiding in this way coarser nerve trunks in the muscle. The Multielectrode was inserted some centimeters proximally and positioned so that it recorded electrical activity from the stimulated fibers. The position in relation to the end plate was determined from the direction of the voluntary action potential over the probe. During the period of searching for optimal recording of single fiber activity the stimulation frequency was held at 0.5-2 impulses/sec. The stimulation strength was kept as low as possible slightly above that value at which some of the stimuli became ineffective.

## *Animal experiments*

Twelve experiments were performed on rabbits. The animals were anesthetized by intravenous injection of sodium pentobarbital (40 mg/kg body weight). Additional doses of sodium pentobarbital were given when needed. In ten experiments the skin and fascia overlying the tibialis anterior muscles in rabbits were carefully cut through and precaution was taken not to damage the superficial layer of muscle fibers. The muscle surface was moistened with a 0.9% saline and kept warm with an ordinary lamp (60 W) at a distance of 20–30 cm.

The muscle was stimulated with two tungsten wires 150  $\mu$  in diameter and isolated except for the sharpened point 5–10  $\mu$  in diameter. The wires were mounted in a micromanipulator and connected to the ordinary output transformer of the stimulators.

As these experiments were carried out in order to investigate whether it was possible to obtain a stimulation such that single fiber activity could be recorded with a Multielectrode, different stimulation techniques were tried and compared. The distance between the wires was changed from 0.5 mm to 50 mm, different stimulus strength and different width of the pulses 60–10 000  $\mu$ sec were used. In some experiments the stimulation was made superficially, in others the wires were pushed into the muscle some millimeters. Recordings were made with the Multielectrode either placed just on the superficial fibers or inserted into the muscle. The procedure was performed under a binocular dissecting microscope with magnification 6–16 times which was also used for inspection of the stimulation result.

*Experiments with curare and sodium citrate.* In order to study the muscle response to electrical stimulation when no indirect stimulation (via the nerve) was possible, two rabbits were given 3 mg of Tubocurarine intravenously (about 1 mg/kg) during continuous stimulation with an intramuscular electrode (DISA 13K14) and recording with the Multielectrode. The animals were artificially ventilated via an endotracheal cannula and kept at room temperature (25–30°C). During the injection the tibialis anterior muscle was studied and when the muscle was totally paralyzed the gastrocnemius, femoral quadriceps, brachial biceps and triceps muscles were also examined.

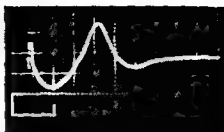
By means of sodium citrate it is possible to elicit electrical activity in nerve and muscle fibers because of its calcium-binding effect. During the total paralysis the 0.1 ml sodium citrate was injected locally in the muscle and the Multielectrode was inserted about 5–10 mm distally to the injection area and the spontaneous activity recorded (cf Ekstedt 1964).

## RESULTS

### *Findings common to both the human and animal experiments*

Two types of electrically evoked responses were recorded from the electrode when it was optimally positioned (Figs 13 and 14 upper left). When the stimulating electrode was inserted distally in the muscle where no nerve fibers were supposed to be present, the most usual response was a coarse action potential with a maximal amplitude of about 30 mV and with a small positive and greater negative peak. This potential had no fast "spike

FIG 13 *Direct muscle response obtained at electrical stimulation of the rabbit muscle during curarization. Calibration 5 mV and 1000  $\mu$ sec*



components" and had a rise time of 0.5–1 msec and a total duration of up to 10 msec. When the stimulation strength was decreased the amplitude gradually diminished to zero. Despite searching for at least 20 experimental hours no recording could be obtained in which a single muscle fiber action potential could be discerned.

When the stimulating electrode was placed more proximally but sometimes also with the same position of the stimulation electrode as above another type of activity was normally recorded with the use of a considerably shorter pulse duration and lower stimulation voltage. There was a complex polyphasic potential form sometimes with up to 30 peaks and a total duration of up to 15 msec, depending on the distance between stimulation and recording needle. When the stimulation strength was decreased the spike components disappeared one by one or a few at a time without interfering with the amplitude of the other spikes. Finally a potential remained in which it was sometimes possible to obtain clean recordings of single fiber action potentials. These potentials did not have a constant time relation to the other part of the complex potential, but varied about  $\pm 10 \mu$ sec.

At high stimulation strength both types of responses could sometimes be seen. The latency of the polyphasic response could in different experiments vary between half and double the latency of the coarse potential.

Sometimes one of the spike components in the polyphasic potential had an opposite impulse direction in relation to the others.

### *Animal Experiments*

The microscopical observation of the muscle during the stimulation with the tungsten wires disclosed that a great part of the muscle was activated even by rather weak stimulation and that small changes in position of the stimulation electrode, some millimeters, changed the twitch considerably.

Under the influence of d-Tubocurarine the complex polyphasic response disappeared completely after an interval of about 5 minutes (Fig. 14) and in this time also the twitch disappeared. When the stimulating electrode was moved in the muscle no response could be obtained with the stimulation

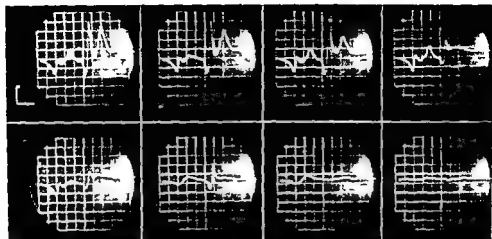


FIG 14 Changes in the electrical response during injection of dTubocurarine into a rabbit. The experiments made in the tibialis anterior muscle. The first recording shown is about 3 min after injection 1 mg/kg the last recording was made 7 seconds later. The components of the polyphasic response disappear and only a coarse response of low voltage and long duration remains. Calibration 2 mV and 1000  $\mu$ s.

parameters used earlier but when the pulse length was increased to 500  $\mu$ sec and the stimulation strength was increased about 3 times a coarse action potential could be obtained which gradually disappeared when the stimulus strength was decreased. The same results were obtained when other muscles (gastrocnemius, femoral quadriceps, brachial biceps and triceps muscles) were examined during curarisation.

When sodium citrate was injected intramuscularly in the curarized muscle, muscle fiber action potentials could be recorded about 10 seconds after the injection. During the first 30 seconds of this activity there was a high impulse frequency after which the activity either stopped or continued at a slower frequency for many minutes.

## DISCUSSION

dTubocurarine has no effect on the muscle fibers but blocks the neuromuscular transmission. It was also certified by means of chemical stimulation that the muscle fibers conducted action potentials normally during curarisation.

The experiments with the use of dTubocurarine have provided evidence that the complex polyphasic type of response obtained by electrical stimulation was caused by nerve stimulation and the coarse potential type (that could only be obtained with the use of higher stimulation voltage and long r

pulse duration) was a result of direct muscular stimulation. This is in close accordance with the findings of e.g. Krnjevic & Miledi (1958) who found that the nerve branches had a much lower threshold than the muscle fibers. The finding that it was not possible to elicit single muscle fiber activity with this type of stimulation technique contrasts with the results in the human brachial biceps muscle obtained by Buchthal, Guld & Rosenfalck (1955a) who have made a claim to have stimulated muscle fibers directly with the same type of stimulation electrode.

When an intramuscular nerve twig was stimulated the whole motor unit was activated. However, even then it was possible to select one of the muscle fibers for recording in the same manner as for recording during voluntary activation.

These facts make it impossible to calculate the exact propagation velocity over a longer distance by means of the latency between stimulus and action potential and the distance between the stimulating and recording needles.



# PROPAGATION VELOCITY DURING PROLONGED ACTIVITY

## SUMMARY

1 The propagation velocity was measured with the Multielectrode technique during continuous voluntary or electrical activation. The brachial biceps, extensor digitorum communis and frontal muscles were investigated.

2 The velocity decreased during activity, the decrease being most pronounced during the first 10 minutes. During this time the velocity on an average decreased in the brachial biceps muscle from 3.37 to 2.92 m/sec and in the extensor digitorum muscle from 3.34 to 3.09 m/sec. The difference in velocity decrease in these two muscles was not significant. Activity from one fiber in the frontal muscle was recorded for more than 10 minutes. The velocity decreased totally 0.01 m/sec during 10 minutes.

3 In experiments with electrical stimulation it was seen that the slope of the decrease curve was dependent on the stimulation frequency. Similar findings were made in the voluntarily activated muscle.

4 When the needle was inserted 30–50 minutes before the activity began, the same slope of the decrease curve was obtained as when the activity started at the time for insertion of the needle.

5 When short periods of rest interrupted continuous activity, the velocity was higher after the pause. This recovery was considered as a normalisation of the velocity.

6 The decrease in velocity was dependent on activity but might have been more pronounced due to the presence of the Multielectrode.

7 The initial velocity was assumed to reflect the propagation velocity in the muscle fiber most closely if no Multielectrode were present.

## INTRODUCTION

Propagation velocity decreases sometimes as much as 50% during 30 minutes of activity. Such a pronounced effect has not been reported in the literature. The aim of this chapter is to study the propagation velocity decrease and some of the factors which influence it.

## METHODS

Propagation velocity was measured with the 1 + 13 Multielectrode. Temperature was recorded in 10 experiments with the thermo needle kept in constant position in the muscle throughout the experiment. The recordings were usually made from voluntarily activated muscle fibers. The experiments were performed on 29 healthy men in the brachial biceps muscle (555 fibers), the extensor digitorum communis muscle (177 fibers) and the frontal muscle (29 fibers). Activity from

a total of 25 of these fibers has been recorded for more than 10 minutes. Despite the fact that the subjects were asked to keep the innervation frequency constant there was a certain irregularity. This made the interpretation of the results more difficult. Therefore some experiments were performed on electrically activated fibers with constant innervation frequency. In the brachial biceps muscle 114 fibers and in the extensor digitorum communis muscle 41 fibers were studied. The fibers recorded were stimulated indirectly via an intramuscular nerve (Chapter 3).

The points in the figures indicate the mean propagation velocity over a 10 second period when not otherwise stated.

## RESULTS

### A Continuous contraction

The activation of the muscle was begun simultaneously with the insertion of the needle and the recording started as soon as the optimal recording position was obtained. Recording was however, not possible during the first 10-30 seconds after the insertion of the needle (the phenomenon of initial silence page 20).

The instantaneous repetition rate of action potentials over a period of 60 seconds was calculated. The coefficients of variation ranged from 8.9 % to 21.5 % in 10 such periods.

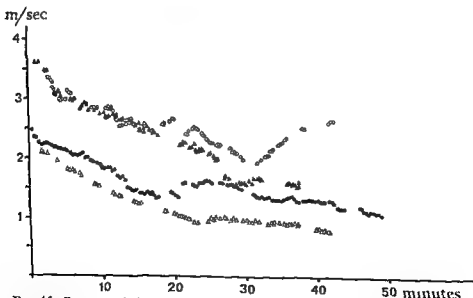


FIG 15 Decrease of the propagation velocity during 40-50 minutes of activity in 4 fibers. Abscissa time. Ordinate Propagation velocity insertion of the Multielectrode at time zero.

The mean repetition rate over the first 10 seconds of every minute was calculated over a period of 30 minutes. The coefficients of variation in 10 experiments ranged from 9.6 % to 22.2 %. The mean frequency at the end of the 30 minutes was the same as at the beginning.

In Fig. 15 results are presented of propagation velocity measurements during continuous contraction. Initially there was a faster decrease and after 20-30 minutes a constant level, about 50 % of the initial, was sometimes reached. Sometimes the velocity decreased still more. In some experiments the velocity, after a normal decrease during 20-30 minutes, began to increase towards initial values.

The different fibers had different initial propagation velocity values and different slopes of the decrease curves (Table 1). There seemed not to be any correlation between decrease rate and initial propagation velocity. Therefore the decrease in Table 1 is given in m/sec. During the first 10 minutes of activity the decrease in propagation velocity appeared to be the same in the brachial biceps and the extensor digitorum communis muscles.

The longest experiment with an individual fiber lasted 3 hours and 17 minutes (about 100 000 action potentials) after which time the recording was intentionally finished. During this time the electrode to fiber distance did not vary more than 15  $\mu$ .

Usually the experiments were stopped earlier due to one of the following reasons:

- a) technical imperfection so that the needle position was changed in relation to the fiber studied because of uncontrolled movements
- b) The action potential being of essentially constant form during many minutes (10-20 minutes) disappeared after a gross change in the potential form during the preceding 30 seconds
- c) The action potential form changed after only a few discharges to a long and low wave with a small negative phase and became impossible to record for more than 1 minute
- d) The potential just disappeared without any warning

## *II Influence of the discharge frequency on the propagation velocity*

To achieve a constant innervation frequency electrical stimulation was used.

At the same frequency the slopes of the velocity curves for different fibers differed considerably. When one and the same fiber was repeatedly stimulated with 2 and 10 pulses per second the curves obtained with each frequency were similar but different for the two frequencies. In Fig. 16 it was shown that the propagation velocity decreased when the fiber was stimulated with 5 and 10 impulses per second. When the frequency after 4-7 minutes

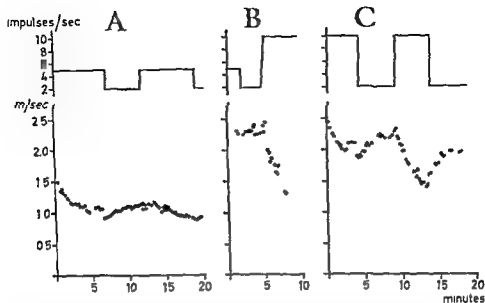


FIG 16 Influence of innervation frequency on the propagation velocity. The muscle fiber was indirectly stimulated via its nerve and recording made with the 1+13 Multi electrode. A Frequency 5/sec gives a decrease of the velocity. Frequency 2/sec here after gives an increase towards the initial value. B and C With the use of stimulation frequency 10/sec the decrease in velocity is greater than when using 5/sec. At 2/sec the velocity increases towards the initial value again.

of continuous stimulation was changed to 2 impulses per second the propagation velocity increased towards the initial values.

The decrease in propagation velocity when changing from a low to a higher stimulation frequency was the more pronounced the longer the stimulation proceeded.

Essentially the same results were obtained with voluntary activation of the muscle. Because of disturbances from other motor units recruited at stronger contraction recordings were usually not possible at innervation frequencies exceeding 15 impulses/sec. Because of the inevitable irregularities in innervation rhythm at voluntary contraction the curves thus obtained have not been subject to a closer analysis.

The results from these experiments showed a correlation between the innervation frequency and decrease in velocity, but it seems likely that other factors than the innervation frequency also influence the decrease in velocity.

### C Effect of pauses

When the activity was interrupted for 50–150 seconds and then resumed it was found in 30 out of 35 cases that the propagation velocity was increased by 0.05–0.4 m/sec after the pause. Effects of resting periods longer than 3 minutes were not studied because of inability to keep the Multielectrode in position for such a long period without fiber activity. After a pause the first registration was not made until the needle was adjusted to optimal position again. After the pause the downward slope was steeper than it had been before the pause (Fig. 17).

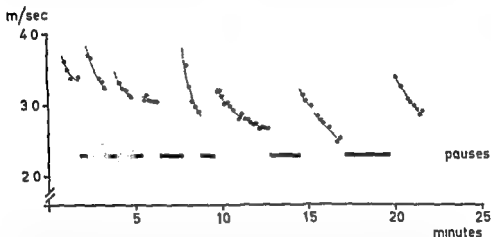


FIG. 17 *The effect of pauses on propagation velocity.* There is a continuous decrease of the velocity. After a pause the velocity is increased in comparison to the value immediately before the pause but there is a more pronounced decrease during the first few seconds of activity. The increase in velocity after a pause seems to be correlated to the length of the pause and to the decrease from the initial value. The duration of the pauses (indicated with heavy lines) were 28, 35, 35, 75, 55, 115 and 150 seconds.

The most pronounced effects of pauses were seen in cases where the experiments had proceeded for many minutes and the velocity in the fiber had decreased.

If the needle was inserted during muscular rest and the activity did not commence until 30–50 minutes afterwards, the velocity decrease curve was of the same type as when the activity started at the time of the insertion of the needle. The slope of the curves during the first 10 minutes (4 cases) 30–50 minutes after the insertion of the needle was in all cases steeper than the slope during the period between the 30th and 40th minutes of activity in any of the curves obtained in the ordinary way. There was no significant difference ( $p > 0.45$ ) in decrease in velocity during the first 3 minutes of activity in the two types of experiments (Fig. 18, Table 1).

m/sec

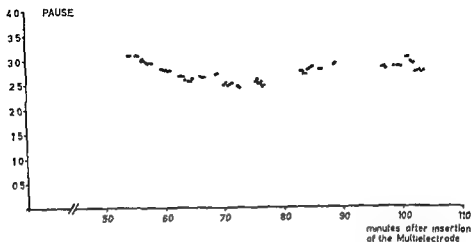


FIG 18 *Initial pause* One of the experiments Activity was started 49 minutes after the insertion of the Multielectrode The decrease in velocity seems to be about the same as when the activity is begun at the insertion of the needle (fig 15)

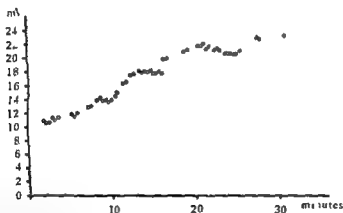


FIG 19 *The action potential amplitude* The first 32 minutes of one of the experiments (totally 38 minutes) presented in Fig 15 (▲)

#### D *Action potential amplitude*

The amplitude of the action potential usually increased during the experiment sometimes as much as 120 % (Fig 19) There seemed not to be any relationship between the decrease in propagation velocity and the increase in action potential amplitude The phenomenon is under further study

TABLE 1

The decrease in propagation velocity in different muscles and after an initial pause

Muscle	Number of fibers	Initial propagation velocity	Propagation velocity after 3 minutes	Decrease in velocity during the first		Decrease in the interval in the first 10 minutes after start of
		m/sec	m/sec	3 minutes	10 minutes	m/sec 10 min
				m/sec	m/sec	
Biceps brachii	10	$3.37 \pm 0.67$	$2.92 \pm 0.63$	$0.45 \pm 0.15$	$1.09 \pm 0.43$ range 0.64-1.82	$0.22 \pm 0.1$ range 0.01
Biceps brachii Activity started 30-50 minutes after the inversion of the Multielectrode	7	$4.03 \pm 0.51$	$3.61 \pm 0.50$	$0.41 \pm 0.17$	$0.47 \pm 0.72$ 0.113	
Extensor digitorum communis	14	$3.34 \pm 0.69$	$3.09 \pm 0.63$	$0.28 \pm 0.15$	$0.85 \pm 0.17$	
Frontalis	1	2.20	2.19	0.01	0.05	

## DISCUSSION

Only a few reports of measurements of propagation velocity in muscle and nerve fibers during prolonged activity have been found in the literature. Halansson (1956) noticed that there was a small continuous decrease in conduction velocity in isolated frog muscle fibers and with a stimulation frequency of about 10 impulses per hour the velocity after two hours was 95-90 % of the value after one hour and 85-80 % after three hours. No figures were given for the decrease during the first 45 minutes of the experiment. He also reported that "with more frequent stimulation there was a more rapid decrease in conduction velocity". The continuous alteration in velocity was ascribed to changes in ion balance when the fiber was kept in an artificial medium. Gerard & Marshall (1933) showed that the propagation velocity in frog sciatic nerve and dog phrenic nerve within a quarter of an hour decreased to an equilibrium level less than two thirds of the normal at a stimulation frequency of up to 300 impulses/sec. The initial values were reached after about 15 minutes of rest. Bullock (1951) reports without further comment that the conduction rate in nerve fibers can decline to 50 % of the normal before failure.

Many factors are known to influence propagation velocity and these might change during activity, but their relative importance in the present experiments cannot easily be predicted

If the change in mean velocity, found with the Multielectrode, were valid for the whole length of the muscle fiber it could be regarded as 'exhaustion'. It is known from the literature that myelinated nerve fibers and squid axons can carry action potentials at high frequency for a long time. When muscle fibers are stimulated with 50-500 impulses/sec they fail to respond with a conducted action potential after one to several seconds. Bergmans (1959) ascribed this to reduction in energy stores but Lüttgau (1964) showed that when the fibers were made non-contractile by continuous stimulation or by metabolic inhibitors the extracellular action potentials showed only little "fatigue" and the fibers behaved like nerves and were able to conduct action potentials over long periods of time at high frequencies. Lüttgau assumed action potential "fatigue" to be due to processes connected with the contraction and its activation.

It has been shown that the membrane potential and internal resistance in frog sartorius muscle change during prolonged stimulation (Schanne, Kern & Schafer 1962). The membrane potential decreased by 12 % and the internal resistance increased by 17 %. It was also shown by Hodgkin & Huxley (1947) that there was a potassium ion leakage from active nerve fibers and that this was associated with changes in membrane properties. In isolated nerve Keynes & Lewis (1951) found a gain in sodium ions and leakage of potassium ions already in the resting fibers and still more in stimulated fibers.

Even if there is support in the literature for the assumption that part of the decrease in velocity found in the present experiment might be present all along the fiber, the results in Chapter 5 showed that a local velocity decrease in the vicinity of the needle cannot be entirely excluded. The following factors could possibly be responsible for this:

- 1) Mechanical damage of the fiber caused by the Multielectrode
- 2) Disturbance of the electrical field by the physical presence of the needle Multielectrode
- 3) Changes in the extracellular ion composition

*Ad 1* The action potentials recorded from fibers lying close to the needle (as judged from amplitude, rise time, voltage decrement curves, propagation velocity) and from fibers at some distance from the needle were in the main of the same shape. If there had been some mechanical damage to the close lying fiber, there ought to have been an "abnormality" in the potential form. Those action potentials described on page 42 as disappearing during the first minute of activity were ascribed to damaged fibers. Such recordings were never used for measurements. Usually several thousands of



action potentials could be recorded before the fiber was lost for one of the reasons mentioned on page 42. It seems likely that the fibers under study were not damaged.

*Ad 2* The presence of the needle might alter the external resistance in the vicinity of the fiber due to its metal shaft or to the surface of the insulator surrounding the electrodes. As the fiber passed the needle approximately in the middle of the epoxy resin area and the distance from the recording electrode to metal was  $500 \mu$  perpendicular to the fiber and  $200 \mu$  along the fiber, it seems reasonable to regard the effect of the needle metal on the electrical field of the fiber as negligible. Because the electrodes are small (the sum of the electrode surfaces occupies about 6 % of the insulator area) their effects as field deformers might also be disregarded. This assumption is also supported by the fact that the "voltage decrement curves" obtained with the two methods in Fig. 5 did not differ (Fig. 8) indicating that the surfaces of the electrodes did not appreciably disturb the electrical field. In the actual experiments the recorded fiber was in the close vicinity of the needle only on one side and over a short distance as the diameter of the needle is 0.6 mm. For reasons of symmetry an insulator covering half the circumference of a fiber might double (but not more) the external resistance. A flat infinite insulator just touching the circumference cannot possibly increase the external resistance very much.

However in this discussion only *changes* in the external resistance are of interest (for discussion of the absolute value see page 50) and a possible effect of the insulator ought to be fairly constant during the course of an experiment.

When the relation between the electrical conductance of the medium and propagation velocity has been studied by different authors (for example Pond 1921) this has been done by changing the chemical composition of the fluid bathing the tissue. The objection could be raised to these experiments that the velocity might have changed as an effect of the alteration in ionic composition independent of the change in external resistance. Hodgkin (1939)

studied the effect of the influence of external resistance upon the propagation velocity by changing the geometry of the volume conductor around the fiber thus keeping the ionic composition constant. When the crab axon was lifted from sea water into paraffin liquid the impulse velocity decreased by about 20 % (12.5–28.8). When a squid axon was lifted from sea water to air the velocity decreased by 53 % (48–58 %). The velocity of the squid giant axon in the whole nerve in situ was approximately the same as when this axon was in a large volume of sea water. It thus seems likely that the disturbance of the electrical field around the muscle fiber due to the presence of the needle has negligible effects on the propagation velocity.

*Ad 3* When the needle was inserted in the muscle, a number of muscle fibers and lymph and blood-capillaries were damaged and the ion composition in the vicinity of the needle changed from normal. As the fiber under study was assumed to be close to the electrode (within  $200\ \mu$  from the electrode surface, cf. Fig. 8) the abnormal extracellular fluid surrounded a part of the fiber and could perhaps change the propagation velocity locally.

Another possibility is that the normal transport of ions and metabolites was disturbed because of the presence of the Multielectrode. This effect ought to be more pronounced at strong activity than at slight.

The extracellular fluid could thus theoretically have changed in two different ways:

- A The ionic composition of the extracellular fluid became abnormal at the insertion of the needle and was gradually normalized during the experiment.
- II The ionic composition of the extracellular fluid was initially normal but altered during the experiment.

#### *A. Abnormal extracellular fluid at the beginning of the experiment*

If the extracellular potassium ions from injured fibers poured out immediately at the insertion, there could theoretically have been an initial depolarisation generating a conducted action potential if the depolarisation was fast or if the depolarisation was slow making the fiber inexcitable (Jenerick & Gerard 1953). The difficulties in obtaining any recording during the first 10–30 seconds after the insertion of the Multielectrode (page 20) might have been caused by such a depolarisation.

If there was an initially changed composition of the extracellular fluid due to the insertion of the needle, this could be assumed to be continuously normalized. The absorption of ions should proceed even during muscular rest although perhaps somewhat more slowly than during the slight activity normally performed during the experiments.

The experiments where activity was not begun until 40–50 minutes after the insertion of the needle indicated that the decrease of propagation velocity did not start until the fiber was activated. This makes it unlikely that the possible change in extracellular ion composition caused initially too high a velocity which continuously decreased toward normal values.

#### *B. Normal extracellular ion composition at the beginning of the experiment*

If the extracellular fluid were normal at the beginning, the propagation velocity decrease ought to be caused by change in the fiber properties or in the composition of the environment around the fiber or to both of these factors. Owing to the presence of the needle, the normal transport of ions

substrate and metabolites around the fiber could be altered because of interference with blood-capillaries and lymph-capillaries and the possible general effect upon the propagation velocity during activity might be enhanced in the vicinity of the needle. Such an effect could be cumulative during prolonged activity. At high frequency the effect ought to be more pronounced than during low frequency. This assumption is supported by the findings in the experiments where the effect of different frequencies was studied and the experiments where the activity was stopped for some seconds after many minutes of activity. If this hypothesis of a change in the extracellular ion composition during activity is true the propagation velocity value measured in the beginning of the activity most closely reflects the velocity as it would have been if no Multielectrode were present.

# PROPAGATION VELOCITY OVER LONG DISTANCE

## SUMMARY

- 1 The brachial biceps and extensor digitorum communis muscles were used
- 2 Attempts to record activity from one and the same fiber with two needle electrodes were not successful neither at electrical stimulation nor at voluntary contraction
- 3 A nerve fiber was stimulated and the latency between the stimulation pulse and the muscle fiber action potentials was measured
- 4 The latency decreased during continuous activity Under the assumption that the nerve propagation time and the neuro muscular transmission time were constant during activity it could be calculated that there was a decrease in propagation velocity not only in the vicinity of the Multielectrode

## INTRODUCTION

In Chapter 4 it was found that the propagation velocity measured with the Multielectrode technique decreased during the course of the experiment in all the fibers and that this decrease seemed to be positively correlated to the degree of activity At rest the velocity recovered The decrease in velocity proceeded most rapidly during the first 15 minutes of continuous activity after which time the velocity reached more constant values an equilibrium was reached between the decrease and the recovery

A decrease in velocity of the order of 30-50 % observed in some of the experiments (Fig 15) has not been reported in the literature There are theoretical reasons for a certain decrease in velocity during continuous activity (page 47) but a local change in the vicinity of the Multielectrode due to its presence cannot *a priori* be excluded

The insertion of the needle might cause alteration of the chemical milieu of the fiber under study so that the values obtained with the Multielectrode would not represent the propagation velocity of the whole muscle fiber

The aim of the present chapter is to investigate whether the observed decrease in velocity is generalized or local

## METHODS

### *Electrical activation*

*One recording electrode* The I+13 Multielectrode was used for recording and a bipolar needle electrode (DISA 13K14) for stimulation The stimulating electrode

was inserted in the distal third of the muscle and the Multielectrode some centimeters proximal to that. The pulse width was 60  $\mu$ sec the stimulation frequency 2-10 impulses/second. By frequent adjustment the stimulation strength was kept at the minimum level at which all stimuli would still be effective (in the following called "threshold stimulation").

Measurements were made of the propagation time of the action potential over the Multielectrode and of the latency between the onset of the stimulus pulse and the base line intersection of the action potential picked up by one of the electrodes in the Multielectrode.

The distance between the stimulating and recording sites was estimated from the distance between the needles along the skin, the depth of the needles in the muscle and the angle between them. The accuracy was not better than  $\pm 2$  mm.

The experiments were made in the brachial biceps muscle (50 fibers in 5 different subjects) and in the extensor digitorum communis muscle (25 fibers in 4 subjects). The subjects were healthy men 25-35 years of age.

*Two recording electrodes.* A technique similar to that described by Buchthal, Guld & Rosenfalck (1955a) was used in about 10 experiments in the brachial biceps muscle. One stimulation electrode (DISA 13K14) was inserted in the distal third of the muscle. One recording electrode (DISA 13K51) was inserted some centimeters proximal to that and the Multielectrode some centimeters still more proximally. A position close to one and the same activated fiber was sought for the two recording needle electrodes.

### *Voluntary activation*

*Recordings from fibers belonging to the same motor unit with two needle electrodes.* The 1+13 Multielectrode was inserted in the muscle and positioned so that the propagation velocity could be measured from one fiber. This action potential triggered the sweep of the oscilloscope. Another recording electrode (usually a concentric needle electrode DISA 13K14, sometimes another Multielectrode) was inserted some centimeters more distally in the muscle and was positioned so that an action potential was recorded which was "tied" to the first potential.

Propagation time was measured both between the potentials recorded from the two needle electrodes and with the Multielectrode.

## RESULTS

### *Electrical activation*

*One recording electrode.* When using one stimulating and one recording electrode the mean value of the time between the stimulus and the muscle response (latency) could vary because of technical factors.

1 By moving the needles in the muscle i.e. changing the distance between the recording and stimulating electrodes, the latency could be changed many milliseconds.

2 By increasing the stimulation strength from threshold value it was possible to shorten the latency up to 1 millisecond.

When however, the needles were kept in a fixed position in the muscle and threshold stimulation was used a change in the latency was still seen during continuous activity

Often many potentials were recorded (belonging to the same motor unit or to different motor units with approximate the same stimulation threshold of the nerve) For technical reasons only the first of the single fiber action potentials in such a complex could be used

In 6 out of 15 cases the latency increased by 1000–3000  $\mu\text{sec}$  (11–23 % of the initial value) during 7–29 minutes In 6 cases the increase was less than 1000  $\mu\text{sec}$  (0–10 %) during 5–12 minutes In one case no change was seen during 8 minutes and in one case the latency decreased 200  $\mu\text{sec}$  (2 %) during 60 minutes of activity

In 2 cases the relative increase in propagation time measured over the Multielectrode was approximately the same as the relative increase in latency but in most experiments far more (Table 2)

There was a variability in the latency between consecutive discharges of the order of 10–30  $\mu\text{sec}$  (SD) The variability of the propagation time over the Multielectrode was of the order of 0.05–0.1  $\mu\text{sec}$  (SD) which is nearly the same as the inaccuracy of the time measurements (page 18)

*Two recording electrodes* In the experiments performed with the technique similar to that described by Buchthal Guld & Rosenfalck (1955a) with electrical stimulation it was possible to record single fiber action potentials from the two recording electrodes. Some recordings were obtained which fulfilled the criteria given by Buchthal Guld & Rosenfalck for measuring propagation velocity namely a) the action potentials disappeared at the same stimulating voltage when it was decreased and b) there was a rectilinear relationship between the arrival time and the distance from the stimulation electrode. However during 50 hours of experiments it was never possible to get a constant interval between the action potentials recorded from the two electrodes

In the experiments with one stimulating and one recording electrode there was a variability in latency of the order of 5–15  $\mu\text{sec}$  at regular stimulation frequency independent of the distance between stimulating and recording electrodes The variability was assumed mainly to be caused by a variability in the neuromuscular transmission time If two electrodes recorded activity from one and the same fiber the variability in latency should be even smaller When the electrode however recorded from different stimulated fibers two motor end plates were involved giving a larger variability In the actual recordings the variability was of the order of 10–30  $\mu\text{sec}$  indicating that the electrodes recorded activity from different fibers

### *Voluntary activation*

In 19 experiments with voluntary activation the recordings were made with two electrodes separated by about 20 millimeters. In all cases (at regular frequency about 30  $\mu$ sec SD, at irregular frequency even more) there was a small variability in the time interval between the potentials recorded from the two needles. This variability seemed usually to be random but in some cases it was influenced by the intervals between the consecutive discharges. In no case during more than 100 hours experiment could it be ascertained (for criteria see page 58) that the two electrodes recorded action potentials from the same fiber, and therefore each experiment was not extended for a long period of time.

## DISCUSSION

### *Direct muscle stimulation*

One way of testing whether the propagation velocity measured over the Multielectrode was valid for a longer distance along the muscle fiber would have been to stimulate the muscle fiber some centimeters from the recording electrode and to measure the time lag between the stimulus and the recorded action potential. Direct stimulation of single muscle fibers proved however not to be feasible (Chapter 3).

### *Electrical stimulation of intramuscular nerve twigs*

*Recording with one electrode* The latency between stimulus and action potentials includes the delay from onset of the stimulus to the initiation of an action potential, the propagation time along the nerve, the neuromuscular transmission time and the propagation time along the muscle fiber. As the distance from the motor end plate to the recording electrode was unknown, as was also the time taken for the impulse to propagate along this part of the muscle fiber, the velocity of the impulse could not be calculated from these experiments. When an "apparent velocity" was calculated this was always higher than the velocity over the Multielectrode. This was partly due to the fact that those action potentials had to be chosen for recording which had the shortest latency. Some other potentials in the response had more than twice the latency of the shortest one.

To get an idea of which apparent length ( $L_{app}$ ) that might correspond to the increase in latency if the propagation velocity was assumed to decrease as much all along the fiber as over the Multielectrode, the following analysis was made. It was assumed that all factors contributing to the total latency were constant except for the propagation time along the fiber. " $L_{app}$ " then was obtained from the following equation

TABLE 2

Measurements of latency between stimulus and an action potential recorded with the Multielectrode and the propagation time over the Multielectrode

Duration of activity	Stimulation frequency	Measurements between two needles			Measurements over the Multielectrode			Distance between the needles	
		Initial latency	Change in latency		Initial prop time	Change in prop time		Actual	L <sub>app</sub>
Minutes	impulses/sec	μsec	μsec	%	μsec	μsec	%	mm	mm
8	5	24 500	3000	12	118	45	38	86.7	15.4
21	5	10 770	1587	15	106	18	17		48
33	2.5-10	10 580	1370	13	91	60	76	40	3.0
7	5	4784	1000	23	57	13	23	20	16
29	5	7900	1000	12				70	
17	1-10	8800	1000	11				38	
21	2.5-10	15 520	850	5.5				60	
5	5	6200	500	8				32	
9	5	6023	440	7	72	71	15		8.8
5	5	6601	18	0.0	111	-24	-21		
6	5	29 350	0	0	150	71	48	104.7	
60	5	10 059	-200	-2					

$$\frac{L_{app}}{L_{ME}} = \frac{\Delta \text{latency}}{\Delta \text{prop time}_{ME}}$$

where  $L_{ME}$  = interelectrode distance in the Multielectrode

$\Delta \text{latency}$  = change in latency and

$\Delta \text{prop time}_{ME}$  = change in propagation time over the Multielectrode

$L_{app}$  was found to be 3 to 48 mm (Table 2). In only one case of 3 where a comparison was possible there was a reasonably good agreement between these values and the actual distance between the needles. In the two other,  $L_{app}$  was much too small. If the propagation time in the nerve and the transmission time in the neuromuscular junction did not change during the experiment these figures indicate either that the impulse had travelled along the muscle fiber for only a short distance and that the decrease in velocity was generalized for this distance or if the length along the muscle fiber was longer than calculated that the decrease in velocity over the long distance was less than that over the Multielectrode. In Fig. 20 one method for calculation of the  $L_{app}$  is presented.

The extreme difficulty of measuring the propagation velocity over the Multielectrode in these experiments must however be stressed. The experimenter had to keep two electrodes: the Multielectrode and the stimulating



impulses/sec



m/sec

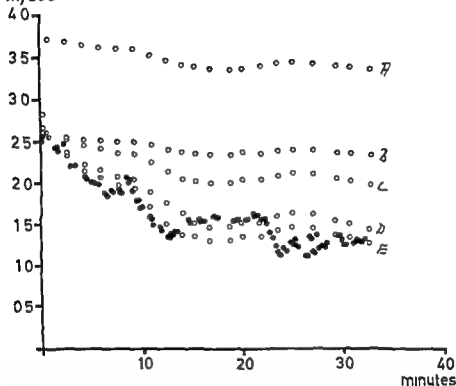


FIG. 20 The propagation velocity decrease over the Multielectrode (D) and the decrease of the apparent velocity over a long distance (A-E). Note that the decrease has a different slope at different stages of the experiment because of different stimulation frequencies. A is the values of apparent velocity that is obtained when the distance between the stimulating and recording needle (40 mm) is used for calculation. In B the propagation distance has been calculated to such a value (277 mm) that the initial velocity is equal to that measured over the Multielectrode. In C, D and E a fixed time (6.9, 9.2 and 9.6 msec respectively) has been subtracted from the latency and thereafter the propagation length calculated to a value that should give the same initial velocity as over the Multielectrode (10.4 and 3 mm respectively). The time subtracted in the three cases should correspond to the propagation time in nerve and the neuromuscular transmission time.

electrode simultaneously in the correct position to within a few micron. Another reason for uncertain results was that the recorded action potential was influenced from other action potentials. This disturbance might change during the experiment when there was a continuous change in the stimulation threshold.

The change in latency was also analysed in the following way. The change in the apparent velocity (total latency divided by that calculated distance which initially gave a velocity of 3.5 m/sec) was compared to the decrease in velocity obtained over the Multielectrode in voluntarily activated fibers given in Table 1. The slope of the decrease curve of the apparent velocity was seen to be approximately the same throughout the experiment. On an average the decrease in apparent velocity expressed as the total decrease during 10 minutes of activity was  $0.25 \pm 0.28$  m/sec which is significantly less than the normal decrease  $1.09 \pm 0.43$  ( $p < 0.01$ , Table 1) obtained over the Multielectrode. When also a constant time value (corresponding to propagation time in nerve and transmission time in the neuromuscular junction) was subtracted from each latency value the relative decrease in apparent velocity was higher than before. However such a calculation cannot be performed with any degree of accuracy because not even a rough estimate of the propagation time in the nerve fiber can be made.

Further factors which could be an influence are in addition. A systematic change in the starting point of the action potentials and continuous change of the propagation time along the nerve fiber and changing neuromuscular transmission time.

The decrease in latency noticed at increasing stimulation strength is a phenomenon that has been reported before and is supposed to be due to a change in the starting point of the action potential (Gerard & Marshall 1933). In the present experiments the stimulation strength was continuously adjusted to threshold and therefore it was assumed that the impulse had started from approximately the same site on the nerve fiber.

A change in neuromuscular delay in skeletal muscle under normal conditions during continuous activity has not been reported in the literature. A decrease in transmission time should partly mask an increased propagation time along the muscle fiber. An increase in transmission time should make the decrease in velocity along the muscle fiber less than calculated. In the present experiments neuromuscular transmission block was never seen (as a sign of the ultimate increase in transmission time).

*Two recording electrodes.* It was never possible to record activity from one and the same fiber with two different electrodes. Therefore the stimulation was not continued for a long period. No further information concerning the

problem of the change in propagation velocity in a single muscle fiber would have been obtained when different action potentials from fibers were recorded with the two electrodes

### *Voluntary activation*

**Two recording electrodes** In these experiments the problem of uncertainty in the starting point on the nerve did not arise. For the calculation of propagation velocity it was however, necessary to ascertain that the electrodes recorded action potentials from the same fiber. This was tested by comparing the short time variation in propagation velocity over the Multielectrode with the variation in "apparent velocity". The propagation velocity for each action potential in a muscle fiber is influenced by the time interval to the preceding impulse (Chapter 6). If the electrodes in the present experiments were recording from one and the same fiber the interval dependence of the propagation velocity should be the same if it was calculated over the Multielectrode or between the two needles. If however the electrodes recorded from two different fibers the interval-dependence in the calculated velocity would not necessarily be the same for the short and long distances. In the two fiber case the propagation time measured between the electrodes is equal to the difference in the conduction time from the common branching point in the nerve to the respective electrode. Theoretically there are several reasons for short time variations in this conduction time

- 1 Uncertainty in propagation across the branching points of the terminal nerve (cf. Krnjevic & Miledi 1958)
- 2 Variability in the propagation velocity in the nerve fiber
- 3 Variability in the neuro muscular transmission time (Ekstedt 1964)
- 4 Variability in the starting point in the motor end plate (del Castillo & Katz 1956)
- 5 Variability in the propagation velocity along the muscle fiber (Chapter 6)

Some of these factors are dependent on the innervation rhythm but the contribution of each factor to the total variability in the latency cannot be predicted. Therefore when the two electrodes were recording from two irregularly innervated fibers belonging to the same motor unit the velocity interval dependence should not regularly be the same when it was measured in one fiber over the Multielectrode as when it was measured between the needles. However even when recording from two fibers this interval dependence might be present provided that there is a considerable difference in propagation length for the two fibers. In this case however the factors 1 to 3 above must be superimposed (page 77) but still the variation in propaga-

tion velocity between consecutive impulses might be similar over the long and the short distance. Thus the presence of the same velocity interval dependence over the long and short distance speaks in favour of a one fiber recording and the absence indicates recording from two fibers. In no case was the same VRF found over the long and short distances.

In this investigation it has been shown that it is extremely difficult to measure the propagation velocity in a single fiber over a long distance *in situ*. As a matter of fact it was not possible in any case to ascertain that the used electrodes recorded action potentials from one and the same fiber in any case. Therefore it has also been difficult to study the change in propagation velocity during prolonged activity. The results indicate that there is a decrease in velocity in the whole fiber during activity, but that the decrease over the Multielectrode is more pronounced.

# VARIABILITY IN PROPAGATION VELOCITY FOR CONSECUTIVE DISCHARGES IN A FIBER

## "THE VELOCITY RECOVERY FUNCTION"

### SUMMARY

- 1 The interspike interval dependent variability in the propagation velocity was studied
- 2 The experiments were performed with voluntary activation or with electrical stimulation of intramuscular nerve twigs
- 3 The general appearance of the interval velocity curve was obtained with double pulse stimulation. For the time interval of about 3-10 msec after a preceding impulse the velocity was *subnormal*. For a longer time interval the velocity was *supernormal* with maximum (112.5-124 %) at the interval  $\approx$  50 msec. After the maximum there was an asymptotic decay. After 500 msec some supernormality still remained. This curve is called the Velocity Recovery Function (VRF).
- 4 When stimulating with a train of pulses the effect of each impulse was added to the remaining effect of the preceding impulses.
- 5 At voluntary contraction where the innervation frequency was irregular this effect causes an interval dependent variability of the propagation velocity.
- 6 A method has been developed for calculating the descending part of the VRF from the interval dependent velocity variations.

### INTRODUCTION

In experiments with voluntarily activated muscles it was seen that the variability in propagation velocity was greater than could be ascribed to inaccuracy in measurements. This variability was studied with the ultimate aim of finding out whether this would give some information about the membrane processes of the muscle fiber under study.

### METHODS

The experiments were performed in the brachial biceps and the extensor digitorum communis muscles in 20 healthy young men. Four different methods were used.

#### 1 Double pulse stimulation

When stimulating electrically the bipolar stimulating needle (DISA 13K14) was placed distally in the muscle and the Multielectrode (1+13) some centimeters more proximally. Square pulses of a duration of 60  $\mu$ sec were used. The strength

of both the conditioning pulse and the test pulse was just above threshold for the nerve fiber (the muscle fiber was usually activated via its nerve Chapter 3) so that spontaneous fluctuations in excitability did not make stimuli ineffective. The interval between the pulses (conditioning to-test interval) was varied and the interval between the different pairs of pulses was always more than 2 seconds.

During the 5–100 msec after the conditioning pulse the nerve membrane was hyperexcitable and therefore the test pulse must be of lower voltage than the conditioning pulse. When using the same stimulation strength for the two pulses usually more nerve fibers were activated by the test stimulus. This was most pronounced when the pulse interval was 5–50 msec. When the interval was less the testing pulse had to be stronger than the conditioning one.

In most cases it was possible to determine from the form of the action potentials whether they were generated from the same fiber or not.

Propagation velocity was measured from the action potential initiated by the test pulses every second of which was preceded by a conditioning pulse.

The time intervals between the conditioning and the test action potentials was measured between the base line intersections of the rising phases. This interval differs somewhat from the interval between the stimuli because of variations in neuromuscular delay, latency in the starting point and different propagation velocities for the two pulses (Farmer, Buchthal & Rosenfalck 1959).

## *2 Stimulation with trains of pulses*

The same general techniques as above were used. The fibers were stimulated with different frequencies in trains with a duration of 10–12 seconds and during the following 30 seconds the fiber was stimulated with a single shock every two seconds. The frequency within the train was 5, 10, 15 or 20 impulses/sec.

## *3 Stimulation with irregular stimulation rhythm*

A comparison of the variability of propagation velocity in different fibers was made by electrical stimulation with an irregular and exactly defined rhythm (programmed stimulation). The experiments were performed in the following way. The discharge rhythm of voluntarily activated action potentials was recorded on a tape recorder (page 19). The tape was played back and the pulses triggered the stimulator. The propagation velocity over the Multielectrode was measured in the ordinary way.

## *4 Voluntary activation*

Propagation velocity was measured with the Multielectrode technique. The subject was asked to make the innervation rhythm irregular. Aided by the sound of the action potentials in the loudspeaker some of the subjects could at will produce certain types of irregular rhythms e.g. a required number of discharges in trains (cf. Hasmaian 1963). The mean frequency was varied from a few up to about 10 per second. At this frequency it was sometimes still possible to record single action potentials without too much disturbance from other motor units. The propagation velocity of each impulse and the interspike interval were determined.

For the analysis of the VRF when the propagation velocity was measured over a long distance ten experiments have been performed according to the

technique on page 51 i.e. the 1+13 Multielectrode recorded activity from one muscle fiber and a concentric needle electrode inserted some centimeters proximally recorded from another fiber belonging to the same motor unit. The recordings were analysed according to the principles on page 68.

The points in the figures indicate the mean propagation velocity over a 10 second period when not otherwise stated.

## RESULTS

### *Double pulse stimulation*

It was found that the difference between the propagation velocity of the action potential elicited by the test pulse and the conditioning pulse was correlated to the interspike interval in a manner shown in Fig. 21. This curve is referred to as the *Velocity Recovery Function* (VRF).

The VRF was different for different fibers and changed during certain experimental conditions (see Chapters 7 and 8). Only five of the VRF curves obtained with the double pulse technique were of reasonably good quality but even these are uncertain because of the experimental difficulties.

A supernormal phase of the VRF was seen in four of the five curves. In

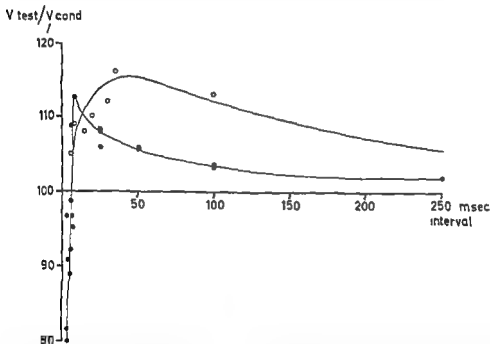


FIG. 21 *Double pulse stimulation*. Two different curves are shown. Abscissa: The time interval between the conditioning and testing action potentials. Ordinate: The velocity of the testing action potential ( $V_{test}$ ) / 100 = the velocity of the conditioning action potential ( $V_{cond}$ ). The curves are fitted by eye. The upper curve is drawn towards a measured value at 500 msec not shown in the figure.

the fifth case no supernormal phase was present. At the end of the refractory period (about 2-4 msec) the propagation velocity of the second action potential was about 20 % lower than that of the first. At 6-10 msec interval the two action potentials had the same propagation velocity. In the four experiments where there was a supernormal phase the curve had a maximum at an interval of 8-50 msec where the second pulse propagated 12.5-24 % faster than the first pulse. The supernormality then gradually decreased. At 50 msec the supernormality was 5, 5, 5.5, 14, 17 and 24 % in six cases and had decreased to 50 % of maximal value when the interval was 100-300 msec. After 500 msec the supernormality was less than 2 %.

### *Trains of pulses*

Thirty fibers in 5 subjects were investigated. When stimulating with trains

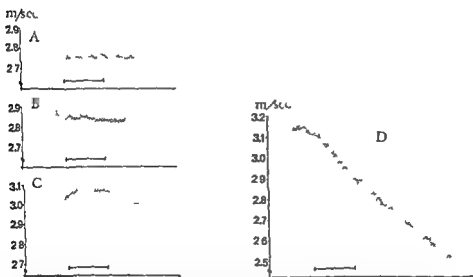


FIG. 22 The effect of stimulation with trains of pulses on the propagation velocity over the 1+13 Multielectrode. The basal stimulation frequency is about 1 per 2 seconds in each case. A 5/sec. Velocity increases 0.05 m/sec. The plateau value is fairly constant. B 10/sec. Velocity increases 0.13 m/sec. The plateau shows a slight tendency to decrease during the train. There is a slight undershoot of the velocity after the train. C 15/sec. The velocity increases 0.33 m/sec (extrapolation because the needle position is not correct during the first 3 seconds). The decrease of the plateau during the trains is pronounced as is the undershoot after the train. For the first second of the pulse train all values are plotted then only every third. D 20/sec. The velocity increases 0.36 m/sec. After 40 seconds of stimulation the velocity has decreased to 2.52 m/sec which is 0.63 m/sec less than the highest value during the train and 0.27 m/sec below the resting value before the train. After the stimulation the velocity tends directly towards the initial value without any further decrease. For the first 3 seconds of the pulse train all values are plotted then only every second.

Calibration in each case 10 seconds



of pulses there was an increase in propagation velocity during the first few seconds from a basal level to higher values which decreased somewhat during the train (Fig 22). The decrease was more pronounced for higher stimulation frequencies than for lower and for each frequency the decrease was more pronounced later in the experiment when the fiber had been stimulated for many minutes. With the stimulation frequencies 5, 10, 15 and 20 impulses/sec the velocity was increased 0.03, 0.15, 0.30 and 0.40–0.60 m/sec respectively. The maximum value was reached after 400–600 msec of stimulation for all frequencies (Fig 22). At the end of the pulse train the velocity decreased as much as it had increased in the beginning of the train i.e. propagation velocity fell below the basal level. After 5–10 seconds the propagation velocity had reached the basal level again.

### *Stimulation with irregular rhythm*

About 20 fibers in 5 subjects were investigated.

Similar results as in the experiments with double pulses were obtained with the irregular innervation frequency. The VRF could, however, not be directly determined as with the double pulse technique. It was not only the last preceding impulse that influenced the propagation velocity but earlier pulses as well. When the same stimulation pattern was used for different fibers the propagation velocity varied in the same manner for the different fibers.

### *Voluntary activation*

The velocity interval dependence could be seen during the experiments by direct observation of the Time Interval Counter where a short interspike interval as a rule was accompanied by a short propagation time. The interval dependence of the propagation velocity has in this way been observed in about two thirds of the voluntarily activated fibers ever since the phenomenon was first noticed, that is to say in many thousands of fibers in the 170 experiments which have been performed.

Closer analysis was made in one of the following two ways:

- The propagation velocity for each impulse was plotted against continuous time. From these diagrams it was possible to determine whether the VRF had a supernormal part (after a short interspike interval the velocity was higher, after a long interval it was lower, Fig 23) a subnormal part (the velocity was lower after a short interval, higher after a long interval, Fig 34 C) or whether the function was near the basal level for the interspike intervals used (no variability in propagation velocity in spite of irregular frequency, Fig 34 B).
- In other cases where at least 200 consecutive discharges and the corre-

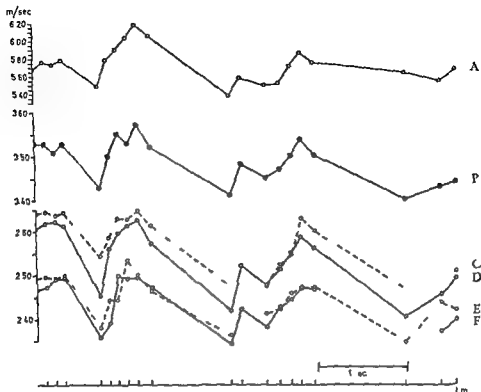


FIG. 23 The velocity interval-dependence at irregular innervation frequency. B is a recording during voluntary activation. The innervation frequency was recorded on a tape recorder which could be played back into the stimulator. D and F shows the variability thus obtained with another fiber in the same subject and with the same stimulation pattern as in B. Both D and F begin approximately with the 350th action potential measured in the respective recording. Action potentials number 1-200 were used for calculation of the Velocity Recovery Function and the propagation velocity values were computed from the time intervals and shown in C and E. Curve A is another fiber.

spending interspike intervals had been recorded and no change in the mean velocity was detected the analysis was made according to the description on page 68.

With voluntary activation time intervals from 50 to 500 msec were normally obtained. Sometimes however even shorter interspike intervals were recorded after a resting period of 3-10 seconds following prolonged activity there were sometimes double discharges with an interspike interval of about 6-10 msec. A similar phenomenon was described by Standaert (1963).

With the analysis described the velocity interval dependence was usually

found in rested fibers but in some cases there seemed only to be a random variability in velocity. No systematic differences could be noticed between the recordings where the VRF did have a supernormal phase and where it did not neither with regard to the slope of the velocity decrease curve during activity, action potential amplitude, innervation frequency, the type of muscle nor to position of the needle in the muscle. With a sweep of  $20 \mu\text{sec}/\text{cm}$  and amplification of  $1 \text{ mV}/\text{cm}$  on the oscilloscope screen the action potential form did not indicate any disturbance from adjacent fibers but nevertheless action potentials from other fibers of the motor unit might have distorted the propagation time interval (Fig. 24)

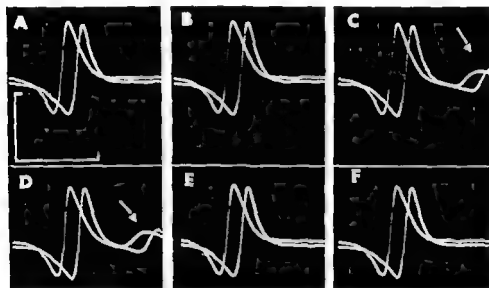


FIG. 24 Interference from another action potential on the propagation velocity measurement. Six consecutive discharges from a long series of recordings. In A, B, E and F the propagation time is  $84.4$ ,  $84.8$ ,  $84.8$  and  $84.5 \mu\text{sec}$  (propagation velocities  $2.855$ ,  $2.842$ ,  $2.842$  and  $2.852 \text{ m/sec}$ ). In C and D an action potential from another motor unit appears (the arrows) in the late parts of the actual action potentials apparently without influencing the potential form but giving propagation times  $86.0$  and  $85.7 \mu\text{sec}$  ( $2.802$  and  $2.812 \text{ m/sec}$ ). Calibration  $2 \text{ mV}$  and  $500 \mu\text{sec}$ .

During continuous activity for a long period the VRF sometimes changed. In some fibers the supernormal phase decreased or even disappeared after 20 minutes of activity. In 2 of 30 fibers the supernormal phase was changed towards a subnormal phase for the normal interspike intervals ( $50$ – $500 \text{ msec}$ ). In none of 50 cases could any changes be detected during the first 10 minutes of activity.

In the experiments with two recording needle electrodes the latency between the action potentials recorded from the two needles varied from  $\pm 25 \mu\text{sec}$  to  $\pm 39 \mu\text{sec}$  (SD) in 7 cases when the interelectrode distance was about 10 mm and from  $\pm 50 \mu$  to  $\pm 70 \mu\text{sec}$  (SD) in 3 cases when the interelectrode distance was about 20 mm. The innervation frequency was irregular. The variability in propagation time over the Multielectrode varied between  $\pm 1 \mu\text{sec}$  and  $\pm 2 \mu\text{sec}$  (SD). An "apparent" velocity between the two needles was calculated. The actual distance between the needles was not used but such a distance as gave approximately the same velocity as that measured over the Multielectrode. The variability in propagation velocity measured both over the Multielectrode and as the "apparent" velocity between the needles was analysed with the method described on page 68. In all of the ten recorded fiber pairs a velocity interval dependence was found both over the Multielectrode and between the two separate needles. Since two fibers were involved in the recording and the real propagation length was not known no quantitative comparison between the variability obtained over the Multielectrode and between the two needles has been made. In Fig. 25 an example

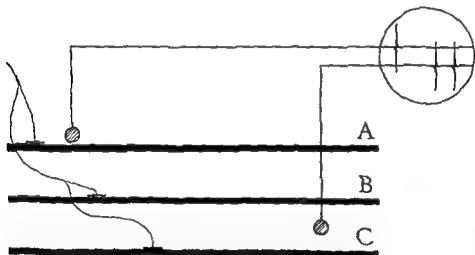


FIG. 25 A recording from three muscle fibers from the same motor unit. Velocity interval dependence was found between action potentials from both fibers A-B and A-C.

of experimental condition is given where one of the electrodes recorded activity from two fibers belonging to the same motor unit.

The amplitude of the action potentials from 50 fibers was studied on photographs but no supernormal phase was found. The amplitude was about 5% lower at an interval of 10 msec and about normal for the testing pulse.

after 300–500 msec. With a train of pulses with 10 impulses/sec the amplitude had decreased to a constant value about 95 % of the maximal after about one second.

### ANALYSIS OF THE VELOCITY RECOVERY FUNCTION (VRF)

As shown in this chapter, the propagation velocity is changed during the recovery phase after an action potential. The general appearance of the velocity interval dependence curve was obtained in the experiments with double pulse stimulation. However, these experiments were technically very difficult to perform and the results uncertain. When the interval between conditioning and testing impulse was changed the threshold of the nerve membrane for the test pulse was also changed. This sometimes caused more than one muscle fiber in the vicinity of the Multielectrode to be activated and the value of the propagation velocity often became erroneous.

Therefore a method for analysis of the VRF from propagation velocity values obtained at voluntary contraction has been worked out.

A period of the experiment was selected where the mean propagation velocity value did not seem to change. From this period (about 10–60 seconds) 200–400 consecutive propagation velocity values with a variation of normally about  $\pm 0.1$  m/sec (range) were taken for analysis.

The whole course of the velocity recovery curve cannot be analysed when using voluntary activation. The interspike intervals (50–500 msec) are only distributed in the descending part of the curve.

An attempt to find a function that fitted the data directly (e.g. an exponential function) was unsuccessful. Therefore the following method was applied.

Since the steepness of the recovery curve seemed to be dependent on the level above a basal value the propagation velocity values were divided into classes with a width of 0.05 m/sec, e.g. 3.00–3.05, 3.05–3.10, 3.10–3.15 m/sec. All action potentials within such a class were regarded as "conditioning pulses". The increase or decrease of the velocity of the action potential arriving after the conditioning action potential was plotted against the time interval between the two. Usually the intervals ranged from 50 to 500 msec. In this way one velocity recovery curve was obtained for each 0.05 m/sec level and all potentials were used once as conditioning, once as test action potentials (Figs. 26 and 27).

The mathematical expression for the biological processes underlying the VRF are not known. To obtain a way of presenting the results and to facilitate further analysis (e.g. when comparing the VRF from different fibers or when the curves in one and the same fiber were studied during prolonged work during ischemia or at different temperatures) a function that suited the data reasonably well was sought from the many possible

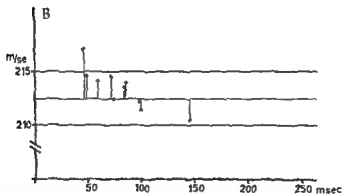
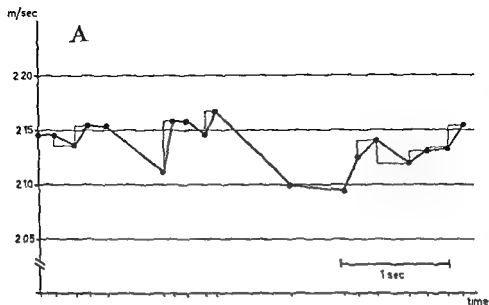


FIG 26 Principles for the analysis of the Velocity Recovery Function at voluntary innervation. The propagation velocity values are divided into classes. In A the values between propagation velocity 2.10 and 2.15 m/sec are thus analysed. All action potentials with propagation velocity within a certain class are considered as conditioning impulses. The change in propagation velocity to the next action potential, the test impulse, is calculated as well as the time interval to this. For each class the change for the test pulse in propagation velocity (abscissa) is plotted against the time interval from the conditioning impulse (ordinate) as made in B. The time scale is different in A and B.

after 300–500 msec. With a train of pulses with 10 impulses/sec the amplitude had decreased to a constant value about 95 % of the maximal after about one second.

### ANALYSIS OF THE VELOCITY RECOVERY FUNCTION (VRF)

As shown in this chapter, the propagation velocity is changed during the recovery phase after an action potential. The general appearance of the velocity interval dependence curve was obtained in the experiments with double pulse stimulation. However, these experiments were technically very difficult to perform and the results uncertain. When the interval between conditioning and testing impulse was changed, the threshold of the nerve membrane for the test pulse was also changed. This sometimes caused more than one muscle fiber in the vicinity of the Multielectrode to be activated and the value of the propagation velocity often became erroneous.

Therefore a method for analysis of the VRF from propagation velocity values obtained at voluntary contraction has been worked out.

A period of the experiment was selected where the mean propagation velocity value did not seem to change. From this period (about 10–60 seconds) 200–400 consecutive propagation velocity values with a variation of normally about  $\pm 0.1$  m/sec (range) were taken for analysis.

The whole course of the velocity recovery curve cannot be analysed when using voluntary activation. The interspike intervals (50–500 msec) are only distributed in the descending part of the curve.

An attempt to find a function that fitted the data directly (e.g. an exponential function) was unsuccessful. Therefore the following method was applied.

Since the steepness of the recovery curve seemed to be dependent on the level above a basal value, the propagation velocity values were divided into classes with a width of 0.05 m/sec, e.g. 3.00–3.05, 3.05–3.10, 3.10–3.15 m/sec. All action potentials within such a class were regarded as "conditioning pulses". The increase or decrease of the velocity of the action potential arriving after the conditioning action potential was plotted against the time interval between the two. Usually the intervals ranged from 50 to 500 msec. In this way one velocity recovery curve was obtained for each 0.05 m/sec level and all potentials were used once as conditioning, once as test action potentials (Figs. 26 and 27).

The mathematical expression for the biological processes underlying the VRF are not known. To obtain a way of presenting the results and to facilitate further analysis (e.g. when comparing the VRF from different fibers or when the curves in one and the same fiber were studied during prolonged work, during ischemia or at different temperatures) a function that suited the data reasonably well was sought from the many possible

The values in each class seemed to be asymptotically approaching a basal level that was roughly the same for the different classes. Evidently they should reach the basal value of propagation velocity that the fiber would have had with a very low innervation frequency. It could thus be anticipated that the function would have the general form

$$V_t = V_{bas} + \frac{B}{F(t)} \quad (1)$$

where  $V_t$  = the actual value of the propagation velocity at time =  $t$

$V_{bas}$  = the basal velocity of the fiber, i.e. the asymptotic value

$B$  = a proportionality factor

$F(t)$  = a function that increases monotonically with time

Different  $F(t)$  were investigated and compared in the following way. In each class a linear regression analysis was made of  $\Delta V$  (difference in propagation velocity between conditioning and testing action potential) on  $1/F(t)$  where  $F(t)$  was  $t$ ,  $\sqrt{t}$  and  $\ln t$ . The linear regression line was calculated with the method of least squares.  $F(t)=t$  and  $F(t)=\sqrt{t}$  did not fit very well. However  $F(t)=\sqrt{t}$  and  $F(t)=\ln t$  both fitted the data well. For comparing the four equations the root mean square difference between the observed values and those calculated was used. In nearly all of 30 examined VRF curves this value was least for  $F(t)=\ln t$  (order of magnitude  $1 \cdot 10^{-2.5} \cdot 10$  m/sec) but the function  $F(t)=\sqrt{t}$  was almost as accurate. The two other functions however, gave values that were 5-10 times higher.

Hence the following equation was used

$$V_t = V_b + \frac{B}{\ln t} \quad (2)$$

However as already said this function does not represent the whole VRF but only the descending part of it and must not be used for values less than about 50 msec (an interval exceeding that where the VRF has its maximum page 63)

In Fig. 28 an example is given from an experiment with voluntary activation of the muscle showing how it was possible to predict propagation velocity values when the spike intervals are known. The VRF-curves for the different classes were determined from 200 consecutive readings. For the following 200 data the propagation velocity of each impulse was read from



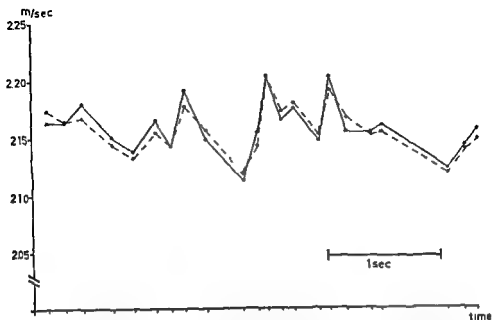


FIG 2 Prediction of the propagation velocity values by means of the VRF. The points joined by lines represent the propagation velocity values of consecutive action potentials (ordinate) plotted against continuous time (abscissa). The VRF was computed for 200 action potentials preceding the shown ones. By means of the function and the interspike intervals the propagation velocities have been computed and shown as the points that are joined by the dashes.

the interspike intervals and the corresponding curves. There was a good agreement between the calculated and actual values.

With the analysis presented two characteristics of the fiber can be obtained: the basal velocity ( $V_{\text{basal}}$ ) and a measure of the supernormality.

Ideally the VRF of a specific fiber would have been that obtained with the double pulse technique. There a conditioning pulse would have a velocity equal to  $V_{\text{basal}}$ . With voluntary activation it was however usually impossible for the subjects to activate the muscle with such a low frequency that  $V_{\text{basal}}$  was approached. Therefore the following procedure was used.

The basal velocity was calculated from each class. Usually these asymptotic values were approximately the same for the different classes in an experiment. Sometimes the  $V_{\text{basal}}$  calculated for the highest class differed from the others by as much as 0.1 m/sec, probably because here the whole curve was based on actual data from very short time intervals. Nonetheless the average of the  $V_{\text{basal}}$  obtained from the different classes was used.

TABLE 3

Parameters of the Velocity Recovery Function calculated according to the method on page 68 For some further normal values see Table 4 (the pre ischemic periods)

Muscle	Experiment and notation	Calculated $V_{\text{basal}}$ m/sec	Calculated increase in propagation velocity at 50 msec for the basal class (per cent)
Extensor digitorum communis	796 Voluntary activity	2.8	3.5
	831 Voluntary activity	2.6	1.6
	Same fiber one minute later	2.6	4.5
	809 After 13 minutes of voluntary activity	2.6	8.5
	Same fiber after 57 minutes of activity	2.6	5.4
	1042 After 1 minute of electrical stimulation	2.3	12.3
	After 12 minutes of stimulation	2.2	8.4
	After 21 minutes of stimulation	2.2	10.7
Biceps brachii	810 After 8 minutes of voluntary activity	2.3	7.1
	After 18 minutes of activity	2.0	-1.8
	823 Voluntary activity	3.9	5.0

A measure of the supernormality was obtained in the following manner. The propagation velocity at an interval of 50 msec for each class was read from the fitted curves and was plotted against the mean value of the classes. A linear relationship was found. As the  $V_{\text{basal}}$  had been obtained as described above, a velocity value at  $t=50$  msec could also be calculated for a basal velocity class. The procedures are presented in Fig. 29. Some of the results are presented in Table 3.

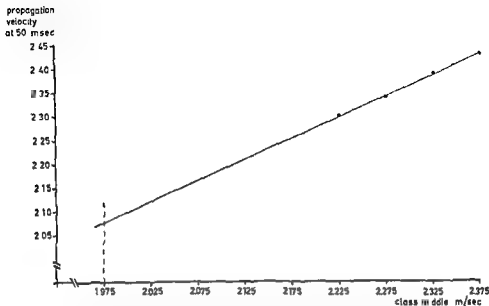


FIG. 29 An attempt to find the parameters for the Velocity Recovery Function for the basal class of the fiber analysed in fig 27. The propagation activity value for base interval 50 msec (see equation 2 page 71) (ordinate) was plotted against the class middle of each Velocity Recovery Curve in fig 27. For the class middle =  $V_{\text{basal}}$  (equation 2 page 71) (i.e. in this case 1.975 m/sec) a 50 msec value of 2.08 m/sec is found.

## DISCUSSION

Changes in the *propagation velocity* of an action potential arriving within the recovery phase of a preceding one have been studied in different excitable tissues by many authors. Gotch in 1910 reported a longer stimulus to response interval for the second impulse than for the first in nerve and proposed that conduction velocity was retarded during the recovery after a previous impulse. At low temperature also a "stimulation fatigue" was assumed to contribute to the extra delay. Lucas (1909-1911) made the same findings in muscle but objected theoretically to Gotch's explanation and concluded that the extra delay depended on other factors unknown at the time than decreased conduction velocity. He introduced the term *Irresponsible Period* for the shortest interval between two electric responses. The experiments of Gasser & Erlanger (1925) in frog's sciatic nerve and in the phrenic nerve of the dog supported the explanation offered by Gotch.

In 1912 Adrian & Lucas reported a *supernormality in excitability* during the recovery phase in frog nerve. In 1920 Adrian showed that the supernormal phase was increased and prolonged with increasing hydrogen ion

concentration Gasser & Erlanger in 1930 found that the supernormality in nerve varied with the after potential. They showed that supernormality of excitability was purely a phenomenon of threshold not of the amplitude of the action potential which decreased during recovery and reached normal height at the end of the refractory period.

Graham (1934) found this close relationship between the after potential and supernormality in excitability and also a *supernormal conduction velocity* in frog nerve. Pillat & Heistracher (1960) found a supernormal phase of velocity in papillary muscle and Farmer, Buchthal & Rosenfalck (1960) made the same observation in human skeletal muscle. However, the entire recovery curve until normal membrane conditions were restored was not investigated and the duration of the supernormal phase has not been determined. Buchthal & Engbæk (1963) studied the supernormal phase of excitability and of conduction velocity in frog sartorius muscle at different temperatures. At 25°C the velocity of an action potential elicited by a test impulse was found to be increased by about 10 % at an interval of 2-3 times the absolute refractory period (the absolute refractory period 2.1 msec). The total duration of the supernormal phase was not determined. Maximal interval between the conditioning and test pulses was 8 times the absolute refractory period. The amplitude of the action potentials was never supernormal during the recovery phase.

The duration of the intracellular recorded negative after potential for the myelinated nerve is 12-20 msec and for the non myelinated nerve 50-80 msec (Grundfest 1940). The time constant of the decay of the after potential in striated frog muscle is about 20 msec (Persson, 1963).

Freygang, Goldstein & Hellam (1964) showed the existence of a *late* negative after potential in frog muscle fiber that cumulated to a mean of 10.6 mV during the first 100 msec of stimulation. After the stimulation it decayed with a time constant of about 300 msec.

One explanation of the negative after-potential in squid axons was offered by Frankenhauser & Hodgkin (1956) who described a small space about 300 Å thick between the excitable membrane and the external solution where effluxing potassium ions were accumulated during the depolarisation due to a net outward flux of potassium which then relatively slowly disappeared into the fiber or into the extracellular compartment. A similar intermediate region has also been proposed for frog muscle (Hodgkin & Horowitz 1960). Its volume was estimated to be 1/500-1/200 of that of the fiber. Freygang, Goldstein, Hellam & Peachey (1964) considered this intermediate space to be the transverse tubular system of the muscle fiber. Here potassium ions were accumulated and depolarized the membrane the late negative after potential. The negative after potential in frog muscle

fibers has been further investigated by Persson (1963) who however concluded that most of the properties of the negative after potential might be explained by permeability changes in the membrane and not by accumulation of ions in an intermediate region outside the membrane

In most of the experiments presented above somewhat unphysiological conditions had to be used e.g. unperfused isolated nerve, stretched muscle fiber (to avoid contractions) or decreased temperature (to slow down the conduction velocity and in this way make time measurements possible). Graham & Lorente de N6 (1938) however, studied the recovery phase of blood perfused rabbit and cat sciatic nerve to obtain more physiological conditions. Contrary to the earlier findings of Graham (1934 1935) no supernormal velocity was found in these experiments and no satisfactory explanation for the divergences could be offered. A supernormal excitability phase was, however, found at least after repetitive stimulation.

It is thus still an open question whether the supernormal propagation velocity is caused by a disturbance in ion balance or by changes in the membrane properties during the recovery period or by other factors.

Any technical faults that might have caused the velocity interval-dependence can at once be excluded (page 31).

Might changes in the fiber diameter (the contraction wave) cause the variation in propagation velocity? This does not seem very likely since the same type of VRF as found in this investigation has been obtained in experiments with stretched fibers in which no contraction wave is present (Buchthal & Engbæk 1963). Also in nerves a similar VRF was reported (see above). Thus the changes in propagation velocity in the recovery phase seems not to be due to changes in fiber diameter during activity.

In the experiments where action potentials from voluntarily activated muscle fibers were recorded with two separate needles it was never possible to record from the same fiber with the two needles. Therefore a direct study of the VRF over a long distance along the fiber was impossible. Some conclusions from the results can nevertheless be drawn.

The latency between the potentials from the two electrodes is equal to the difference in propagation time from the common branching point in the nerve to the electrode. Each of these propagation times might vary mainly because of a) variation in the propagation time in the nerve b) variation in the neuro muscular transmission time and c) variation in the propagation time in the muscle fiber (page 58).

When the two fibers were recorded with two electrodes separated by many millimeters the action potentials travelled different distances along the muscle fibers. A supposed variation in propagation time in the muscle fiber ought to contribute to the variability in the total latency.

In the experiments where the distance between the electrode needles was about 20 mm, the variability in latency was 50–70  $\mu\text{sec}$  where the inter-electrode distance was about 10 mm the variability was 25–39  $\mu\text{sec}$  and in the experiments reported by Ekstedt (1964) where the distance between the recording electrodes was very small (about 200  $\mu$ ) the variability (the jitter) was 10–30  $\mu\text{sec}$ . Even here the action potentials might have travelled different distances along the muscle fiber (depending on the distance between the motor end plates). Part of the jitter might therefore still be ascribed to the variability in propagation time in the muscle fiber.

The finding that the jitter was considerably greater when the interelectrode distance was increased seems to exclude the possibility that the VRF is a local phenomenon over the Multielectrode.

The results from this investigation indicate that the same type of changes in the recovery phase as found in other excitable tissues is also present in the human muscle fiber *in situ*.

## PROPAGATION VELOCITY DURING ISCHEMIA

## SUMMARY

The effect of ischemia on propagation velocity and on the velocity recovery function was studied

1 The extensor digitorum communis muscle was made ischemic by means of a blood pressure cuff with or without previous application of an Esmarch roller

2 During ischemia the velocity could decrease (5 out of 23 cases) increase (3 out of 23 cases) or be unchanged (15 out of 23 cases) in relation to the pre ischemic slope of the velocity curve

3 The velocity recovery function was unchanged in 4 out of 20 cases It lost the supernormal phase in 10 out of 20 cases and in 6 out of 20 cases the supernormal phase changed to a subnormal

4 The influence of the innervation frequency upon the propagation velocity became continuously more pronounced during the period of ischemia

## INTRODUCTION

The effect of ischemia on propagation velocity can obviously not be investigated on isolated muscle fibers because these are already deprived of their circulation and oxygenated by perfusion The influence of pure anoxia on the muscle fiber has been studied earlier but the combined effect of anoxia and arrested blood circulation (with prevention of normal removal of metabolites and ions) on the propagation velocity seems not to have been investigated

The present chapter is aimed at elucidating the effect of arrested blood circulation on the mean propagation velocity and on the Velocity Recovery Function (VRF) in the muscle fiber

## METHODS

The investigation was performed on 9 healthy men 25-35 years old in the extensor digitorum communis muscle This muscle is fairly easy to keep slightly contracted for a long time It is made ischemic more easily than e.g. the brachial biceps muscle

The experiments were performed in one of the following ways

A In most of the experiments a blood pressure cuff was placed loosely around the upper arm The muscle was voluntarily activated by lifting a finger and the Multielectrode was inserted During the first 10-15 minutes 10-100 readings

of the propagation time of consecutive action potentials were made every 10 seconds to define the pre ischemic slope of the decrease in velocity. For the study of the Velocity Recovery Function (VRF Chapter 6) 1-5 records with 200-400 consecutive readings each were made when the innervation frequency was made very irregular. The interspike intervals ranged approximately from 50 to 500 msec.

The cuff was then insufflated to 200-250 mm Hg and the subject was asked to keep the same innervation frequency as before ischemia. During the ischemic period the propagation velocity was recorded as before. Now and then the innervation frequency was made irregular for the study of the VRF.

The circulation was restored after maximally 40 minutes, sometimes earlier due to the inability of the subject to activate his muscle. The desufflation was made slowly over a period of half a minute because otherwise it was impossible to keep the Multielectrode in position. Despite this precaution the fiber was usually lost. In 10 experiments the same fiber was still recorded after the desufflation of the cuff. In the cases where the recording from a fiber was finished a new fiber was sought and the recording was commenced as soon as possible in order to follow the changes in mean velocity in the postanoxic period.

*B* In some experiments the muscle was activated by electrical stimulation and the propagation velocity measured over the Multielectrode. The latency between the stimulating pulse and the action potential was also recorded.

*C* In 3 experiments bloodlessness was aimed at. The ischemia was induced by winding an Esmarch roller from the fingers to the axilla. The blood pressure cuff was applied and insufflated the roller was removed and the needle was inserted and the recordings made according to one of the above described ways. With this method the same fiber could not be investigated before and after the application of ischemia. The measurements and other methodological details were the same as those described in Chapter 1.

In 2 of the experiments the intramuscular temperature was measured. The same technique as described in Chapter 1 was used.

## RESULTS

### *General findings*

A few minutes after the ischemia was applied the subject felt paraesthesia for some minutes beginning peripherally and reaching the wrist. The subjects normally felt increasing pain after 10-15 minutes. In some cases a sensation of cold was reported after 10 minutes of ischemia. In all experiments the subjects noticed a muscular weakness which was often accompanied by an involuntary compensatory increase in the innervation frequency.

During the first few minutes following the restoration of circulation the subject felt the same type of paraesthesia as during the induction of ischemia, usually more pronounced the longer the period of ischemia. During the first minutes in the post ischemic period spontaneous multiple firing was often recorded. The action potentials were generated from one fiber and appeared in bursts with 2-6 potentials in each. The time interval between



each discharge was 8–20 msec and they seemed to be triggered by voluntarily activated action potentials. The types of activity were the same as found during manifest and latent tetania (see 1a Isch, 1963). In some cases fasciculations were seen and in one subject there was a cramp in the forearm for approximately one minute. The reactive hyperemia was accompanied by a sensation of warmth.

These general findings were in accordance with those of Kugelberg (1944).

### *Propagation velocity*

The effect of ischemia upon the mean propagation velocity varied considerably between the different experiments. In 3 cases however, where the same fiber was recorded during two or more periods of ischemia separated by a period of 10–15 minutes with restored circulation essentially the same type of change in velocity was found during the different ischemic periods (Fig. 33).

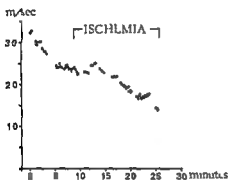


FIG. 30 The effect of ischemia on propagation velocity. During ischemia the velocity seems to decrease in relation to the expected course of the curve.

In 5 out of 23 periods of ischemia the slope of the velocity decrease curve became steeper after some minutes of ischemia (Fig. 30). In 4 out of 23 experiments there was a tendency to an increase in velocity during the first minutes of ischemia (as seen in Fig. 31).

In 15 out of the 23 experiments no significant change could be detected between the slopes of the pre ischemic and ischemic curves (Fig. 32). In 3 of the experiments the velocity increased during the whole period (10, 10 and 12 minutes) of ischemia (Fig. 33).

Usually the mean innervation frequency was about 10 impulses/sec. As reported in Chapter 4, the propagation velocity decrease curve became steeper when the innervation frequency was increased. The influence of innervation frequency upon propagation velocity became continuously more pronounced during the period of ischemia.

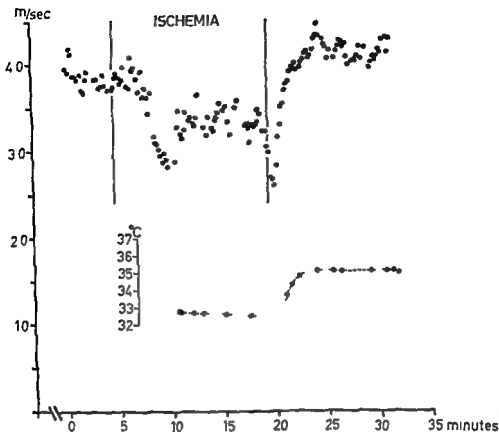


FIG 31 Influence of a period of ischemia on propagation velocity. The velocity is shown to decrease during the ischemic period. Immediately after restoration of circulation the intramuscular temperature rises and propagation velocity increases. Each value of propagation velocity is the mean value for all action potentials occurring during a 10-second period.

In the 14 experiments where it was possible to measure the propagation velocity directly after the restoration of the circulation a transitory increase in mean velocity was seen in 3 cases. The increases were 30 %, 31 % and 35 % above the value at the end of the ischemic period and reached a maximum after 4–5 minutes (Fig 31). The velocity then decreased again.

#### *The Velocity Recovery Function*

The propagation velocity values were treated in one of two ways according to the principles given on page 64 for the study of the VRF: the velocity values were plotted against continuous time or the changes in propagation velocity from the preceding impulse was plotted against interval (Figs 34 and 35).

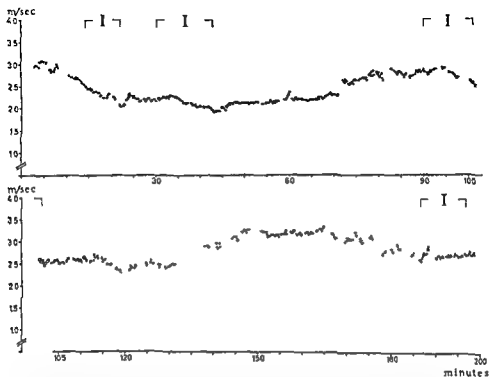


FIG 32 A continuous recording of propagation velocity during 3 hours and 17 minutes. Each value is the mean value of at least half - usually all - the propagation velocity measurements during 10 seconds. Ischemia has been applied during four periods (I) in all of which the velocity decreases.

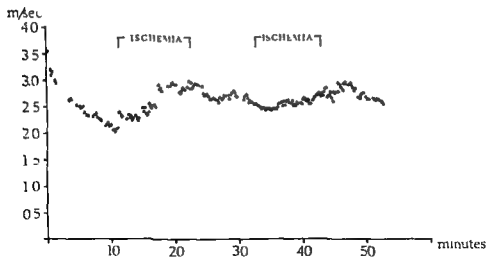


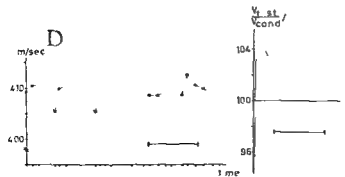
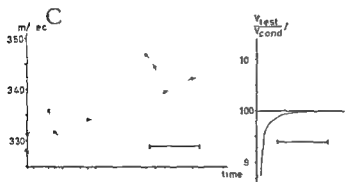
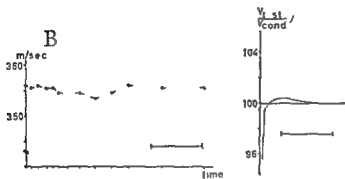
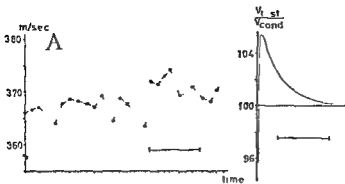
FIG 33 The effect of ischemia on propagation velocity. The propagation velocity increases during both the ischemic periods.

In the pre ischemic period the VRF was of the general form as described on page 63 in all of 20 fibers. In 4 of these cases there was no gross change of the supernormal part during 7, 12, 19 and 30 minutes of ischemia. In 10 cases the supernormal part disappeared after 3, 5, 6, 6, 8, 10, 10, 13, 15 and 20 minutes of ischemia but no subnormality was seen. In 6 cases however the VRF was changed from the normal appearance to that shown in Fig. 35 with a subnormal phase after 4, 8, 9, 13, 13 and 25 minutes. Ten minutes after restoration of the circulation the VRF seemed to be normalized in 2 out of 3 cases. To clarify the magnitude of the changes in VRF some of the results are presented in Table 4.

TABLE 4

Changes in the parameters of the V-velocity Recovery Function during ischemia

Experiment Number	Time Minutes	Notation	Calculated $V_{\text{basal}}$ m/sec	Calculated propagation velocity increase at 50 msec for the basal class (in percent)
1016	0		2.4	4.7
	2		2.1	4.2
	4		2.0	5.2
	5		1.9	8.2
	7	Start of ischemia		
	8		2.1	4.8
	9		2.3	3.4
	12		2.3	2.3
	18	End of ischemia		
	19		2.6	1.6
	22		2.4	6.2
	23		2.4	4.9
	24		2.3	3.6
	29	Start of ischemia		
	30		2.3	5.5
	31		2.3	6.2
	33		2.2	6.9
	36	End of ischemia	2.3	6.2
1044	0		2.6	4.7
	1		2.5	4.7
	2		2.5	4.6
	3		2.4	4.6
	4		2.3	2.4
	10	Start of ischemia		
	12		2.1	-1.0
	14	End of ischemia	2.2	-1.2



## Temperature

During 2 of the experiments the temperature was also recorded. During the ischemia the intramuscular temperature decreased  $1^{\circ}\text{C}$  during 20 minutes in one case  $0.5^{\circ}\text{C}$  during 30 minutes in another. After the relief of ischemia the temperature increased  $1.5$  and  $2.6^{\circ}\text{C}$  respectively during the first 5 minutes, and was thus above the pre ischemic level. After another 5–10 minutes it slowly began to return to the initial value.

## DISCUSSION

The main findings concerning the propagation velocity during ischemia were: The slope of the velocity decrease curve was usually steeper than normal but sometimes no change was detectable. In 3 cases there was even a slight increase in velocity.

A transitional increase in velocity was sometimes seen when the ischemia was applied and when the circulation was restored.

For a given change in the mean innervation frequency the propagation velocity changed more at the end of the period of ischemia than at the beginning.

The Velocity Recovery Function (VRF) usually changed heavily in shape during ischemia and returned to its normal appearance after a short time when the circulation was restored.

Due to the variations in the obtained results no general conclusion concerning the effect of ischemia on propagation velocity could be drawn. Since in experiments without ischemia occasional variations in the velocity of the same order of magnitude are found as during ischemia even rather obvious changes concomitant with the ischemic period have to be analysed in relation to normal variations. Sometimes ischemia seemed to induce a somewhat steeper slope of the velocity decrease curve. The reason for this effect might be that the factors normally causing the velocity decrease are strengthened or that new factors are added. If the normal decrease in propagation velocity

---

FIG. 34 Influence of ischemia on the velocity interval-dependence. *A* Diagram to the left is Velocity Time Diagram i.e. the variation of propagation velocity (ordinate) plotted against continuous time (abscissa: calibration line 1 sec). To the right is illustrated a possible form of the Velocity Recovery Function not calculated. Ordinate: Propagation velocity of the testing action potential ( $V_{test}$ ) in relation to the conditioning ( $V_{cond}$ ) in per cent. Abscissa: Interval between conditioning and testing action potential. Data from the pre ischemic period. *B* After 10 minutes of ischemia the supernormal phase of the VRF is diminished. *C* After 14 minutes of ischemia the VRF is inverted and is subnormal in its whole course. *D* 4 minutes after restoration of circulation the VRF is normalized.

were related to an accumulation of metabolites restricted circulation might have the effect seen in the present experiments

The reason for the great variability in effect of ischemia upon the velocity might be either a) different inherent characteristics in the fiber or b) different conditions for the fibers in the different experiments

a) Different fibers might have a different energy consumption during activity which might explain the varying effect of ischemia upon them. Another possibility is that a greater membrane surface of a fiber makes it more sensitive to disturbances in normal circulation. The present data did not allow any statistical analysis of the correlation between the propagation velocity (as an arbitrary measure of the fiber diameter see page 98) and the amount of decrease during ischemia. However the experiments where the propagation velocity of one and the same fiber responded to ischemia in the same manner during different periods of ischemia, indicate that inherent factors are responsible for most of the observed differences between the fibers.

b) If the region around the fiber under study had been badly perfused (because of the normal variations of local circulation in the muscle) during the time preceding the ischemia this might have strengthened the effect.

During periods of ischemia there was in many experiments a tendency to an increase of the mean innervation frequency when the subject was not repeatedly ordered to keep it constant. In the present experiments however the mean frequency was kept constant within approximately the normal range of variations during the whole experiment. Furthermore there was no essential difference in the curves obtained with electrical stimulation and voluntary activation and therefore the change in velocity during ischemia cannot be ascribed to changes in innervation frequency.

The increase in propagation velocity during the first minutes following the application of ischemia and during the first minutes after restoration of ischemia might be of the same nature as described for nerve by Kugelberg (1944) Magladery MacDouglas & Stoll (1950) and Gilliatt & Willison (1963). These authors considered the increase in velocity to be due to a lowered threshold of the membrane.

The consistent finding of an increasing influence of the innervation frequency on the propagation velocity during ischemia has also been reported by Bullock (1951). This might be due to a superimposed effect on the decrease in basal velocity of an inverted VRF. When the VRF has a subnormal part in the time interval of 50-500 msec (the limits for the intervals usually appearing in the voluntarily activated muscle Fig. 35) an increase

in innervation frequency will decrease the mean velocity and accentuate the change in velocity

The change in VRF during ischemia was a fairly constant finding. In cases where the ischemic period was longer than 15 minutes there were obvious changes in the shape of the VRF (Fig. 35). Corresponding changes were not seen during continuous activity for more than 30 minutes without ischemia. When the supernormal part changed to a subnormal for the actual interspike intervals (50–500 msec), a stage was passed in which the curve was flat and the propagation velocity fairly constant for consecutive discharges even at irregular frequencies. In some cases, however, the curve seemed to pass through some more complex form not exactly known before the subnormal part was seen. During this intermediate stage no correlation was found between interval and change in velocity.

The change in VRF during ischemia might be responsible for at least part of the change in mean velocity in the following way. A mean innervation frequency of 10/sec (which is a common value in the experiments) was assumed. With a normal VRF the mean velocity would be increased by about 5 % above the basal level. With the VRF inverted by ischemia (Fig. 35) the velocity would be decreased by about 5 % below the basal level at the same innervation frequency. The continuous change of the VRF during an experiment might thus change the velocity from 105 % to 95 % of the basal, i.e. a decrease by about 10 %.

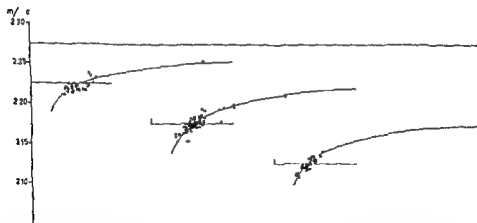


FIG. 35 *Inverted Velocity Recovery Function after 9 minutes of ischemia.* For explanation of the principles for drawing of the figure see fig. 27. The horizontal line in the upper part of the figure is the asymptotic line which the three curves are approaching.



The increase in velocity, seen in the early postanoxic period might be ascribed to the normalisation of the VRF, but still other factors could theoretically be responsible for this effect. One such factor is the increased temperature that was actually observed in the experiments. Another factor is an increase in excitability such as has been reported to occur in nerve (page 86).

The fact that it was impossible in most cases to record from the same fiber before, during and after the period of ischemia makes the results somewhat more difficult to interpret. The main conclusions, however, concerning the propagation velocity and the VRF seem not to be affected by this fact. Ischemia seems to influence the mean propagation velocity to only a minor degree, much less than the effects of changes in innervation frequency. The VRF is, however, entirely changed in most of the fibers.

Actual measurements of propagation velocity in muscle fibers during ischemia have not appeared in the literature. However, the findings presented above are in general agreement with what was known earlier about the effects of anoxia.

Farmer, Buchthal & Rosenfalck (1960) reported a change in the recovery cycle in human muscle fibers during ischemia. The refractory period was prolonged from 2.2-4.6 msec to 5.0-6.2 msec.

In isolated muscle fibers from the frog the after depolarisation (negative after potential) has been shown to be more sensitive to change in the electrolyte milieu (Macfarlane, 1952) and in metabolic activity (Macfarlane & Meares, 1955, 1958a) than the spike and the resting potential. In experiments with anoxia Macfarlane & Meares (1958a) found that the after potential in frog muscle fibers was reduced within 20 minutes at which time the action potential was still mainly unchanged. The after potential recovered within 10 minutes of the return of oxygen.

The effect of anoxia upon the resting and action potentials and after potentials in nerve fibers has been investigated by many authors (for references see Macfarlane & Meares, 1958a). Essentially the same results as those obtained with muscle fibers were reported. The propagation velocity in whole nerve *in situ* was measured by Gilhott & Willison (1963) who found an increase of 12 % during the first 3-5 minutes of ischemia and during the same period an initial increase of excitability, followed by a decrease.

The fact that a change occurs in a muscle fiber parameter (the VRF) under ischemic conditions might prove of value in the further study of muscular disorders and the effect of some pharmacological agents on muscle fibers.

# PROPAGATION VELOCITY AT DIFFERENT TEMPERATURES

## SUMMARY

1 The intramuscular temperature of the brachial biceps muscle was changed between 22.1 and 39.0°C by alteration of the external temperature of the upper arm

2 In relation to the normal change in propagation velocity during activity an increase in temperature up to 39°C seemed to increase the propagation velocity in all of 6 cases. When the temperature was decreased by 9°C significant changes were seen in 2 out of 5 cases. When the temperature was decreased by 4°C in 3 cases no change was seen

3 An increase in temperature from normal did not alter the Velocity Recovery Function in the one case where this was studied. A decrease of the normal temperature below 30°C made the supernormal phase disappear in the 3 cases where this was studied

4 The variation in intramuscular temperature when the environmental conditions were reasonably constant were small and does not appreciably influence the propagation velocity or the Velocity Recovery Function

## INTRODUCTION

Only a few reports are found in the literature concerning the propagation velocity and temperature. Wilska & Varjoranta (1940) found a nearly linear relationship between temperature and propagation velocity in the muscle fibers of the abdominal muscle of the frog. When the temperature was increased from 10 to 36°C the velocity increased more than five times. In isolated frog muscle in whole muscle preparation Buchthal & Engbæk (1963) investigated the velocity in the temperature range of 14–25°C and found a positive correlation with a  $Q_{10} = 1.6$ . In intact human muscle Buchthal & Rosenfalck (unpublished data cited by Buchthal & Sten Knudsen 1959) found a temperature coefficient ( $Q_{10}$ ) of 2 in the range of 30–37°C. Between 26 and 30°C there was no significant change in propagation velocity with temperature. In human nerve the velocity decreased at decreasing temperature (see Gassel & Trojaborg 1964).

The propagation velocity during the recovery phase following an action potential has not been studied with regard to its dependence on the temperature in muscle fibers. In nerve Tasaki (1949) found that the supernormal

velocity in the recovery phase disappeared and that the refractory period increased at cooling

The experiments presented in this chapter were made in order to evaluate the effect of temperature upon the propagation velocity and on the Velocity Recovery Function

## METHODS

The experiments were performed in the brachial biceps muscle in 4 healthy young men. The muscle was voluntarily activated with a slight contraction. The general methods for recording and for the measurements of the temperature is described in Chapter 1. The thermo needle was placed about 1 cm from the Multielectrode at the same depth from the surface.

The experiments did not begin until several minutes after the insertion of the Multielectrode and thermo needle. During this time the temperature differences between the needles and the surrounding tissue were equilibrated.

The temperature was changed in the muscle by means of the following device. A 1.5 mm thick rubber tube 1 cm in diameter loosely twisted round the upper arm about 20 turns. The tube was connected to a water tap with warm or cold water. The Multielectrode and the thermo needle were inserted between two turns of the tube, care being taken that there was no contact between needles and tube. In this way the temperature in the biceps muscle could be changed from 39°C (pain threshold) to 22.1°C (below which temperature no action potentials could be obtained).

## RESULTS

### *General findings*

*Spontaneous temperature changes* When the thermo needle was placed in different positions in the muscle and there was no water in the tube the temperature was found to vary within the muscle by about 2°C. The lowest values were obtained near the surface.

Some minutes after normal activity had ceased the temperature slowly decreased. At slight contraction, however, the temperature was constant within 0.8°C for a time period of 30 minutes.

*Artificially changed temperature* When the temperature in the tube was changed the intramuscular temperature in the vicinity of the thermo needle began to change within one minute. The temperature increased approximately 0.5–1.6°C per minute and decreased 0.2–0.8°C per minute within the temperature range of 28–37°C.

At low temperature below 30°C the subjects often had a feeling of weakness and when the temperature was decreased below 27°C occasional drop out of potentials was noticed (a single action potential in a sequence of otherwise regular discharges would be lacking). The total duration and the rise time of the action potentials increased in gross proportion to the

decrease in velocity The amplitude of the action potentials was usually lower than normal In one experiment the temperature was 22.1°C Here no activity could be elicited with voluntary contraction but with electrical stimulation action potentials with low amplitude and a slow rise time were recorded

At high temperature (39°C) no gross change in the potential form was seen The innervation frequency and rhythm was normal

### *Propagation velocity*

*Decreased temperature* Activity from about 20 fibers were recorded but as the experiments had to go on for more than 10–15 minutes recordings from only 5 fibers could be used for analysis The other fibers were lost because of some of the factors described on page 42 or because of difficulties in activating the muscle fibers when the temperature was low In 2 out of 5 cases the velocity decrease curve became significantly steeper when the temperature was decreased about 9°C (from 39°C to 30°C and from

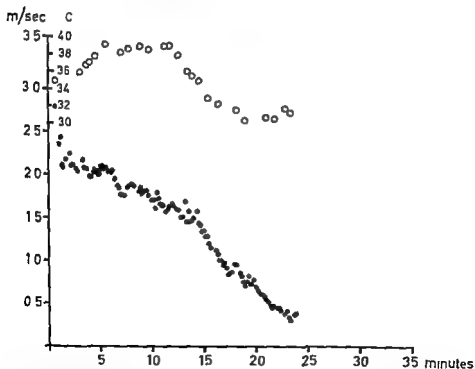


FIG. 36 The effect of temperature on propagation velocity Abscissa continuous time Ordinate intramuscular temperature (upper) and propagation velocity Note the time lag between the change in temperature and change in propagation velocity

*Increased temperature* In one experiment the temperature had been kept at 29°C for 12 minutes during which time the VRF had lost the super normal phase. After this period the temperature was raised to 35°C within 7 minutes. The VRF was continuously normalized and after 5 minutes at about 34°C there seemed to be a normal velocity interval dependence. When the temperature in one experiment was 39°C for 10 minutes no gross changes in the VRI from normal were found.

## DISCUSSION

Although the material is small it seems to be possible to draw some conclusions from these experiments concerning the effect of temperature upon the electrical activity of the muscle fiber. The propagation velocity and the VRF in all the investigated fibers were influenced by changes in temperature in a reproducible way, but no quantitative analysis has been made.

There was a positive relationship between temperature and the propagation velocity in the temperature interval of 23–39°C. Below 23°C no action potentials could normally be elicited and 39°C was the maximal intramuscular temperature obtained with this technique before the subjects felt too much pain.

When the intramuscular temperature was made to increase after a period of continuous decrease or the reverse there was usually a certain delay of 1–10 minutes before the propagation velocity responded to the change. This delay could not be ascribed to error in measurements of temperature i.e. different depths of the thermo-electrode and the Multielectrode (the same delay was seen when the thermo electrode was inserted 0.5 cm deeper than the Multielectrode as when it was 0.5 cm nearer the surface). The cause of this delay is not known.

The cause of the dependence of propagation velocity on temperature was not further studied. Experimental errors such as change in mean innervation frequency or ischemia due to pressure of the water tube can be excluded. The delay mentioned above would seem to indicate that metabolic factors are involved.

Since ion movements are involved in bioelectrical phenomena it seems reasonable that a lower temperature slows down the rate of these processes. The effect on the propagation velocity is however much more complex shown for example by Macfarlane & Mearns (1958b). They showed that the rate of rise of the intracellular action potential (in frog sartorius muscle) increased with temperature but that the voltage of the action potential decreased. The same findings have been made by Hodgkin & Katz (1949 b) in the giant axon of the squid. These two changes influence the propagation

velocity in opposite directions (Hodgkin & Katz 1949a) Another example showing the complex influence of temperature is given by Fitzhugh (1966) in a theoretical analysis of the Hodgkin Huxley (1952) nerve model. He found the stimulation threshold temperature curve to be U shaped for short stimulating pulses but monotonically increasing for long pulses.

The effect of different temperatures upon the VRF with a decrease in the supernormal phase at cooling is in accordance with what was found in the motor nerve of the toad by Tasaki (1949). He found that the recovery processes slowed down and that the supernormal phase disappeared between 12°C and 6°C. The change of the recovery phases in the muscle fiber has not been studied earlier. The negative after potential, however, which is correlated to the supernormality in velocity (page 75) was found to decrease at low temperatures (Macfarlane & Meares 1955, 1958) in frog sartorius muscle and to increase in toad sartorius muscle with increasing temperature (Ishiko & Sato, 1957). Corresponding figures are reported for nerve (Lorente de No 1947).

From the practical point of view the conclusions can be drawn that the intramuscular temperature variations at constant environmental temperature are small and cannot appreciably alter the propagation velocity and Velocity Recovery Function.

# PROPAGATION VELOCITY IN DIFFERENT MUSCLES

## SUMMARY

- 1 The propagation velocity was measured in different human muscles *in situ*
- 2 The mean velocity in the voluntarily activated brachial biceps muscle in twelve healthy subjects was  $3.69 \pm 0.71$  m/sec ( $n=443$ ). The same value was found with electrical stimulation
- 3 There was a positive relationship between the circumference of the upper arm and the mean propagation velocity in the brachial biceps muscle
- 4 There were differences in mean propagation velocity in different muscles e.g.  $2.01 \pm 0.39$  m/sec ( $n=26$ ) for the frontal muscle  $3.15 \pm 0.75$  m/sec ( $n=147$ ) for the extensor digitorum communis muscle and  $3.39 \pm 0.68$  m/sec ( $n=142$ ) for the femoral quadriceps muscle
- 5 In one case of disuse atrophy of one of the femoral quadriceps muscles the mean propagation velocity was significantly lower in this muscle than in the normal

## INTRODUCTION

Earlier experiments indicate (page 11) that there exists a correlation between propagation velocity and fiber diameter both in isolated nerve and muscle fibers. In human muscle only a few measurements of propagation velocity have been made *in situ*. Only Buchthal, Guld & Rosenfalck (1955b) have analysed the velocity diameter relationship and proposed that for the fiber *in situ* the diameter influences propagation velocity only to a minor degree.

In this chapter propagation velocity (measured within a defined time interval after the insertion of the needle) of a number of muscle fibers in different subjects and in different muscles in the same subject was measured. An attempt was made to correlate the propagation velocity with the fiber diameter but this study was not aimed at finding out the mathematical equation for the relation between these parameters, but at getting a preliminary view of propagation velocity values in different fibers and to compare different muscles. Some experiments on pathological muscles were also performed.

## METHODS

This study was made at an early stage of the investigation and the wobbler technique (page 17) was used in most of the experiments. The 3+11 Multi-electrode was used.

The muscles were voluntarily activated at the same time as the needle was inserted. After the necessary corrections of the needle position usually finished within 30 seconds, the propagation velocity was recorded for many minutes. The mean value of 5-15 measurements during the 3 minutes following the initial 30 seconds was taken as a measure of the propagation velocity of the muscle fiber. A new needle position was sought for each fiber recorded.

In experiments on normal muscles 443 fibers were measured in 12 normal brachial biceps muscles, 147 fibers in 7 extensor digitorum communis muscles, 26 fibers in two frontal muscles, 142 fibers in two femoral quadriceps muscles. In other experiments 49 fibers in brachial biceps muscles from 2 children were studied.

One atrophied and one trained femoral quadriceps muscle in one patient were examined (72 and 42 fibers respectively).

In one series of experiments 92 fibers in 2 brachial biceps muscles were electrically stimulated (2-5 impulses/sec) and the propagation velocity measured with the Multielectrode technique.

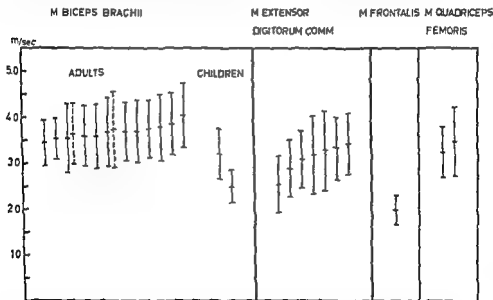


FIG. 39 Propagation velocity in different muscles. Abscissa: different samples in the different muscles arranged in ascending order of propagation velocity. Ordinate: propagation velocity  $\pm 1$  SD. The samples which are marked with dashes have been obtained with electrical stimulation from the respective subject.

## RESULTS

The mean propagation velocity in samples of muscle fibers in the brachial biceps muscles in healthy subjects varied from  $2.48 \pm 0.34$  m/sec in a



child (with a circumference of the upper arm of 16.6 cm) to  $4.04 \pm 0.70$  m/sec in a well trained subject (circumference 32.6 cm). The mean value for the whole material (adults) was for the brachial biceps muscle  $3.69 \pm 0.71$  m/sec ( $n=443$ ) for the extensor digitorum communis muscle  $3.15 \pm 0.75$  m/sec ( $n=147$ ) for the femoral quadriceps muscle  $3.39 \pm 0.68$  m/sec ( $n=142$ ) for the frontal muscle  $2.01 \pm 0.39$  m/sec ( $n=26$ ). The mean velocity in the brachial biceps of two children, 4½ and 6½ years old was  $2.48 \pm 0.34$  m/sec ( $n=10$ ) and  $3.23 \pm 0.56$  m/sec ( $n=39$ ) respectively. In both cases the differences from the normal material is highly significant ( $p < 0.01$ ). See Fig. 39.

As to the subjects where the circumference was measured (7 cases) there was a positive correlation (Fig. 40) between the circumference of the upper arm and the mean velocity in the muscle ( $p < 0.05$ ).

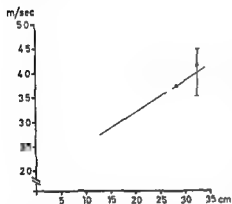


FIG. 40 Propagation velocity in the brachial biceps muscle plotted against upper arm circumference. The regression line calculated with the method of least squares (<sup>2</sup>) indicates the mean value for a sample in a case of acromegalia (not used in calculation of the regression line). The line through that value indicates the standard deviation for the normal material at this circumference.

The results of the measurements with the Multielectrode after electrical stimulation were within the range of the values obtained from the voluntarily activated normal muscles.

One patient had his right knee joint immobilized for 3 weeks in a plaster cast after an operation upon a meniscus (Fig. 41). One month after removal of the plaster the first measurements were made in his quadriceps muscles. In the normal leg (circumference 43.5 cm, 13 cm above the patella) there was a mean velocity of  $3.48 \pm 0.74$  m/sec ( $n=82$ ). In his operated leg (circumference of 39.5 cm) the mean velocity was  $2.78 \pm 0.36$  m/sec ( $n=38$ ). The difference between the mean values is significant ( $p < 0.01$ ) and also the difference in standard deviation (variance ratio 4.2,  $p < 0.01$ , Moroney 1960). One year later the mean velocity in the normal leg (circumference of 42.3 cm) was  $4.23 \pm 0.55$  m/sec ( $n=42$ ) and in the quadriceps muscle formerly immobilized (circumference of 40.8 cm) the velocity was

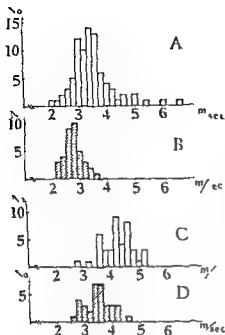


FIG. 41 *Effect of disuse atrophy on propagation velocity* See page 98. Frequency distribution of samples of propagation velocity in both quadriceps femoris muscles

*One month after removal of the plaster cast*

- A Normal left leg
- B Atrophied right leg

*One year later*

- C Normal left leg
- D Atrophied right leg

$3.49 \pm 0.48$  m/sec ( $n=34$ ) The difference between the mean values is significant ( $p < 0.01$ ) but not the difference in standard deviation

## DISCUSSION

It is known from literature (cf. page 11) that there is a correlation between the diameter of the isolated muscle fiber and its conduction velocity in vitro. For human muscle fibers in situ however Buchthal, Guld & Rosenfalck (1955 *a, b*) found the velocity in voluntarily activated muscle to be  $4.72 \pm 0.54$  m/sec ( $n=26$ ) and for electrically activated fibers  $4.02 \pm 0.43$  m/sec ( $n=16$ ) despite the fact that fibers were known to range from 25–85  $\mu$  in diameter in the normal biceps muscle. This small variability in velocity was ascribed to a high external resistance due to the arrangement of the fiber in subunits. The fiber under study and those around it were all considered to be activated synchronously and from the point of view of the actual fiber the external resistance seemed to be high acting to minimize the influence of diameter.

In the experiments described in this chapter it was generally found that mean values of propagation velocity during the period 30–210 seconds after the insertion of the needle ranged from for example 1.5 to 5.8 m/sec in the same muscle (mean  $3.67 \pm 0.75$  m/sec  $n=159$ ). The mean values

were less in small muscles (the frontal muscles) than in coarse (the femoral quadriceps and brachial biceps muscles) The found correlation between the circumference of the upper arm and propagation velocity in the biceps muscles might indicate a relationship between fiber diameter and propagation velocity

The mean velocity as defined in this chapter was about 13 % lower than it would have been if the velocity had been measured at the time for insertion of the needle (Table 1) As the decrease in velocity (in m/sec) did not seem to be correlated to the initial velocity value, the range of measured velocity was mainly the same as if the velocity had been measured at the time for insertion of the needle

Another question that arises when measuring a sample of muscle fibers with this method is whether there is any overrepresentation of potentials from large fibers because of their higher amplitudes or of small fibers because there are more small fibers than large ones per unit muscle area To little is known about the volume conduction of the currents in the muscle to answer this question Is there any biological bias in these experiments when the muscle is only slightly activated? It is known that smaller nerve fibers have a higher electrical threshold than larger ones but no reports are found in literature as to whether there is any difference in mean value of muscle fiber diameter between motor units with large and those with small nerve fibers It is not known which motor units are recruited first at the slightest contraction It must therefore be stressed that the present experiments with voluntarily activated fibers are made on samples of fibers recruited at slight effort

The absolute values of propagation velocity were somewhat lower than at zero time because the fiber had worked for 3 minutes As no difference in this decrease has been detected in different muscles, the comparisons between the mean velocity could be made

The present experiments speak in favour of a positive correlation between velocity and fiber diameter

## SOME FINDINGS IN MUSCULAR DISORDERS

## SUMMARY

- 1 The mean value of propagation velocity was lower than normal in one case of disuse atrophy and in two cases of dystrophia myotonica
- 2 The propagation velocity was within the normal range in 5 cases of myxoedema
- 3 In one of two cases of acromegalia the mean velocity was significantly higher than in healthy subjects
- 4 The mean propagation velocity seemed to decrease during thyroid therapy in a case of myxoedema
- 5 The Velocity Recovery Function was shown to be changed in myxoedema but seemed to be normal in dystrophia myotonica and myasthenia gravis

## INTRODUCTION

During the course of the study of propagation velocity in human muscle fibers a few patients with muscular disorders have been investigated to give an idea whether the method might be of value in the study of muscular diseases

## METHODS

The same general methods as described in Chapter 1 were used  
The 3+11 and 1+13 Multi-electrodes were used

## RESULTS

*Propagation velocity*

In a patient already described on page 98 and in Fig. 41 with a mild atrophy in his quadriceps femoris muscle due to inactivity there were significant differences between the mean values of propagation velocity in samples of muscle fibers from the atrophied and normal leg. One year later the circumference of both legs had increased and the propagation velocity in the muscle fibers in both quadriceps had increased as well.

In 5 patients with *myxoedema* the propagation velocity values in the exten-

sor digitorum communis muscle were within the normal range. The values given for adults in Chapter 9 are used as normal values ( $3.15 \pm 0.75$  m/sec,  $n=147$ ). In one of the patients measurements in a sample of muscle fibers in the extensor digitorum communis muscle were made before and 1 and 2 months after beginning of thyroid therapy. The mean velocity decreased from  $3.73 \pm 1.05$  m/sec ( $n=10$ ) to  $3.05 \pm 0.78$  m/sec ( $n=13$ ). The difference between the mean values is probably not significant ( $0.05 < p < 0.1$ ). Evidently more fibers have to be investigated.

In two patients with *dystrophia myotonica* with an obvious muscular atrophy the mean velocity in the brachial biceps muscle was  $2.52 \pm 0.72$  m/sec ( $n=11$ ) and  $2.42 \pm 0.30$  m/sec ( $n=6$ ) which is significantly lower than in healthy subjects ( $p < 0.01$ ).

Two patients with *acromegalia* were examined. One had a significantly higher mean propagation velocity in the brachial biceps muscle than normal  $4.15 \pm 0.87$  m/sec ( $n=26$ ,  $p < 0.01$ ). However this mean velocity falls on the linear regression line of the propagation velocity on the circumference of the upper arm in normals. One had a mean propagation velocity in brachial biceps within the normal range  $3.63 \pm 0.70$  m/sec ( $n=16$ ,  $0.3 < p < 0.4$ ). The circumference was not measured.

### *Velocity Recovery Function*

In myxodema the VRF of 10 of 20 fibers had an abnormally small supernormal phase. In 2 cases a subnormal phase was seen (for the intervals 50–500 msec) and this was never seen in healthy subjects. In 2 cases however the supernormal phase seemed to be more pronounced than normally seen at an interval of 50 msec the velocity was increased by 30 %.

In two cases of *dystrophia myotonica* about 20 fibers and in five cases of *myasthenia gravis* about 15 fibers were investigated. The shape of the VRF did not seem to differ from the normal.

## DISCUSSION

In ordinary electromyography the motor unit activity is the subject of study and the compound action potentials from several fibers are recorded. The method used in this investigation however gives information about the single fiber.

The finding of a decrease in mean propagation velocity in the muscle with *disuse atrophy* is of interest as this type of atrophy cannot be diagnosed by ordinary EMG.

The smaller standard deviation of the propagation velocity values from the atrophied muscle and the fact that the lower limit of the distribution did

not fall below that in the normal leg might indicate that the larger fibers had decreased more in diameter than the smaller ones. Further study is needed.

The high mean velocity values in the patients with *acromegalia* can probably be ascribed to the large fiber diameter which these patients are known to have.

In *myxoedema* there are different muscular symptoms of which the slowed relaxation is most common. The muscle fibers are swollen as a result of accumulation of mucinous substances. Electromyographic changes in the form of reduction in size and duration of the motor unit potentials and increased proportion of polyphasic potentials are reported (e.g. Åström, Kugelberg & Müller 1961). The first patients with myxoedema were investigated on the basis of the question whether the propagation velocity was lowered when the basal metabolic rate was low. All the 5 patients, however, were found to have the propagation velocity within normal limits. In one patient studied before as well as during therapy, the velocity showed a tendency to decrease during treatment. The propagation velocity is thus not decreased (because of the low metabolic rate) but might be increased because of the fiber swelling. This requires further study.

The membrane processes underlying the change in velocity in the recovery phase are dependent on metabolic factors (page 88). The change in the VRF in the patients with *myxoedema* might be an effect of changed metabolic activity in the muscle fiber.

The velocity was decreased in the 2 cases of *dystrophia myotonica*. Whether or not this decrease can be ascribed to the decrease of the muscle fiber diameter or to other factors as well cannot be settled at present. The present analytical treatment for the VRF did not indicate any difference from the normal. However, in muscular dystrophies it is known that there are changes in the properties of the muscle fiber membrane (Farmer, Buchthal & Rosenfalck 1959; Norris 1962). Therefore the mathematical analysis of the VRF is undergoing further development to make it more sensitive to detect changes that could be present in this disease.

## GENERAL SUMMARY

The propagation velocity of single muscle fiber action potentials in voluntarily activated human muscle *in situ* has not earlier been studied. The present monograph is aimed at developing a method for such measurements and at studying the influence upon the propagation velocity of some factors.

In *Chapter 1* the methods are described. The method is based on the use of a needle Multielectrode. In the side of the needle (0.6 mm in diameter) are mounted 14 platinum electrodes, each of diameter 25  $\mu$ . The electrodes are arranged in a pattern that permits the recording of single muscle fiber action potentials from one and the same fiber with two electrodes at a mutual distance of about 250  $\mu$  along the fiber. The time lag between action potentials so recorded (of the order of magnitude of 50–200  $\mu$ sec) is used for calculation of the propagation velocity.

Because of the small distances and short time intervals that are measured it is necessary that the needle Multielectrode be correctly positioned in relation to the fiber to obtain reliable results. In *Chapter 2* criteria for correct position are discussed and an analysis made of the errors in measurement. The maximal errors in the measurement of the propagation velocity were thus found to be +4 % and -2 %. Slow variations could occur during an experiment due to unnoticed position changes giving errors of the order of magnitude of  $\pm 1$  %. The variations in propagation velocity between consecutive measurements that are caused by technical errors can not have exceeded 0.5 %.

It would have been valuable to compare the propagation velocity over a longer distance of the muscle fiber with the values obtained over the 250  $\mu$  distance in the Multielectrode. An attempt was therefore made to stimulate electrically single muscle fibers *in situ*. In *Chapter 3* the results of these experiments are presented. It was not possible to restrict the stimulation to only one or a few muscle fibers, either when using a needle electrode in the human muscle, or when using sharpened tungsten wires in the rabbit muscle. The stimulation always gave a response in a great many fibers. However, it was possible to obtain a more or less polyphasic potential in which single

muscle fiber action potentials could be discerned and recorded. This type of response disappeared after injection of d-tubo-curarine in the rabbit and was therefore considered to have been caused by stimulation of nerve twigs. One or several motor units must thus have been activated.

In *Chapter 4* the results of propagation velocity measurements during long continuous voluntary activity are reported. The velocity was always found to decrease from the initial values most pronounced during the first 10 minutes. In the brachial biceps muscle (initial mean propagation velocity  $3.37 \pm 0.67$  m/sec SD) the velocity decreased  $1.09 \pm 0.43$  m/sec (SD) during the first ten minutes and in the extensor digitorum communis muscle (initial mean propagation velocity  $3.34 \pm 0.69$  m/sec SD) the decrease was  $0.85 \pm 0.37$  m/sec (SD) during the first ten minutes. There was no difference in rate of decrease between fibers with high or low initial propagation velocities. There were also slow variations in propagation velocity up and down. The innervation frequency was shown to influence the rate of decrease of propagation velocity: at low frequencies the decrease was slower than at high frequencies. After a pause in activity the values were higher than immediately before the pause. When the Multielectrode was inserted 30–50 minutes before the activity began, the velocity decreased similarly to the curves obtained in the normal way.

The following question now arose: Are the changes in velocity that were shown with the Multielectrode technique representative for the whole muscle fiber, or are they only local changes in the vicinity of the needle caused by the mere presence of the needle? This problem was dealt with in *Chapter 5*. Direct stimulation of a muscle fiber could not be made as shown above. Attempts were made to record the activity from the same fiber with two needle electrodes, but this failed. Therefore experiments with stimulation of a nerve fiber and recording from its muscle fiber were made. The experiments seemed to indicate that the decrease in propagation velocity over the Multielectrode was mainly but not exclusively a local phenomenon. Further investigations are needed.

When analysing the propagation velocity measurements it was found that the variability in velocity was greater than could be ascribed to technical errors. In *Chapter 6* this variability was studied and was found to be dependent on the time interval between the action potential discharges. When the time interval from the preceding action potential was short (below 5–10 msec) the velocity was lower than for the preceding action potential; when it was longer the velocity was supernormal with a maximum of up to 25% at 8–50 msec. A method was developed for the analysis of this *Velocity Recovery Function* from the data obtained at voluntary contraction with the



## GENERAL SUMMARY

The propagation velocity of single muscle fiber action potentials in voluntarily activated human muscle *in situ* has not earlier been studied. The present monograph is aimed at developing a method for such measurements and at studying the influence upon the propagation velocity of some factors.

In *Chapter 1* the methods are described. The method is based on the use of a needle Multielectrode. In the side of the needle (0.6 mm in diameter) are mounted 14 platinum electrodes, each of diameter  $25\ \mu$ . The electrodes are arranged in a pattern that permits the recording of single muscle fiber action potentials from one and the same fiber with two electrodes at a mutual distance of about  $250\ \mu$  along the fiber. The time lag between action potentials so recorded (of the order of magnitude of 50–200  $\mu$ sec) is used for calculation of the propagation velocity.

Because of the small distances and short time intervals that are measured it is necessary that the needle Multielectrode be correctly positioned in relation to the fiber to obtain reliable results. In *Chapter 2* criteria for correct position are discussed and an analysis made of the errors in measurement. The maximal errors in the measurement of the propagation velocity were thus found to be +4 % and -2 %. Slow variations could occur during an experiment due to unnoticed position changes giving errors of the order of magnitude of  $\pm 1\%$ . The variations in propagation velocity between consecutive measurements that are caused by technical errors can not have exceeded 0.5 %.

It would have been valuable to compare the propagation velocity over a longer distance of the muscle fiber with the values obtained over the  $250\ \mu$  distance in the Multielectrode. An attempt was therefore made to stimulate electrically single muscle fibers *in situ*. In *Chapter 3* the results of these experiments are presented. It was not possible to restrict the stimulation to only one or a few muscle fibers either when using a needle electrode in the human muscle or when using sharpened tungsten wires in the rabbit muscle. The stimulation always gave a response in a great many fibers. However, it was possible to obtain a more or less polyphasic potential in which single

## ACKNOWLEDGEMENTS

This work was carried out in the Department of Pharmacology University of Uppsala. To the head of the Department Professor Ernst Barany I express my sincere gratitude for his stimulating advice and for his penetrating and constructive criticism which has been of inestimable value.

The investigation has been made in close collaboration with Docent Jan Elstedt whom I wish to thank most sincerely for his constant, unselfish help.

My thanks are also due to Docent Lars Erik Larsson, Docent Sigfrid Blom and Docent Karl Erik Hagbarth for many valuable discussions.

Fk Gunnar Gruvæus has been consulted for some of the problems concerning the numerical analysis of the results. For this I am greatly indebted.

I am especially grateful to the staff of the department to Miss Sig Britt Lindström, Mr Leif Andersson and Mr Gösta Loven for technical assistance, to Mr Per Haggqvist for construction of the Multielectrodes, to Research Engineer C Ivar Sjöberg and to Civil Engineer Jan Czekajewski for construction of many of the electronic devices, to Mr Manne Fredriksson for the photography and many other services, to Mrs Ann Mari Kjellberg and Mrs Kerstin Sundström for secretarial aid and to Mr Torsten Ekwall for preparing many of the diagrams.

I thank the staff of the Uppsala Data Center for their help.

I would like to express my thanks and admiration for those who patiently acted as experimental subjects.

I cannot sufficiently thank my wife Eva for her encouraging support and great patience throughout the entire investigation.

Financial support has been provided by the Swedish Association Against Poliomyelitis, the Magnus Bergwall Foundation, the Swedish Staff Pension Society, the Medical Faculty of Uppsala University and the Swedish Medical Research Council (Projects Nos 14X-135, 14X-559).

## REFERENCES

- Adrian E D* The recovery process of excitable tissues *J Physiol (Lond)* 1920 54 1
- Adrian E D & D W Bronk* The discharge of impulses in motor nerve fibres Part II The frequency of discharge in reflex and volutary contraction *J Physiol (Lond)* 1929 67 119
- Adrian E D & A Lucas* On the summation of propagated disturbances in nerve and muscle *J Physiol (Lond)* 1912 44 68
- Astrom K E Kugelberg & R Muller* Hypothyroid Myopathy *Arch Neurol* 1961 5 472
- Basmaian J V* Conscious control of single nerve cells *New Scientist* 1963 20 662
- Beránek R* Intracellular electromyography in man *Physiol Bohemoslov* 1961 10 94
- Bergmans J* Effect d'une contraction tetanique prolongee sur l'activite electrique du muscle strie *Arch Int de Physiol* 1959 67 538
- Buchthal F & L Engbaek* Refractory period and conduction velocity of the striated muscle fibre *Acta physiol scand* 1963 59 199
- Buchthal F C Guld & P Rosenfalck* Propagation velocity in electrically activated muscle fibres in man *Acta physiol scand* 1955a 34 75
- Innervation zone and propagation velocity in human muscle *Acta physiol scand* 1955b 35 174
- Buchthal F P Høpke & J Lindhard* Temperature measurements in human muscles *in situ* at rest and during muscular work *Acta physiol scand* 1944 8 230
- Buchthal F & O Sten Knudsen* Impulse propagation in striated muscle fibers and the role of the internal currents in activation *Ann N Y Acad Sci* 1959 81 422
- Bullock T H* Facilitation of conduction rate in nerve fibres *J Physiol (Lond)* 1951 114 89
- del Castillo J & B Katz* Localization of active spots within the neuromuscular junction of the frog *J Physiol (Lond)* 1956 132 630
- Creutzfeldt O* Membranpotentiale in menschlichen Muskeln bei gesunden Personen und Patienten mit Adynamia episodica hereditaria und periodischer Lahmung *Naturwiss* 1962 49 284
- Denslow J S & C C Hasett* The polyphasic action currents of the motor unit complex *Amer J Physiol* 1943 139 652

- Eccles J C & W J O'Connor* Responses which nerve impulses evoke in mammalian striated muscles *J Physiol (Lond)* 1939 97 44
- Ekstedt J* Human single muscle fiber action potentials *Acta physiol scand* 1964 61 Suppl 226
- Ekstedt J & E Sjöberg* A method of recording extracellular action potentials of single muscle fibres and measuring their propagation velocity in voluntarily activated human muscle *Bull Amer Ass EMG Electrodiagn* 1963 10 16
- Farmer T W, F Buchthal & P Rosenfalck* Refractory and irresponsive periods of muscle in progressive muscular dystrophy and paresis due to lower motor neuron involvement *Neurology* 1959 9 747
- Refractory period of human muscle after the passage of a propagated action potential *EEG Clin Neurophysiol* 1960 12 455
- Fitzhugh R* Theoretical effect of temperature on threshold in the Hodgkin Huxley model *J Gen Physiol* 1966 5 989
- Frankenhauser B & A Hodgekin* The after-effects of impulses in the giant nerve fibres of toad *J Physiol (Lond)* 1956 131 341
- Freygang W H Jr, D A Goldstein & D C Hellman* The after potential that follows trains of impulses in frog muscle fibers *J Gen Physiol* 1964 47 929
- Freygang W H Jr, D A Goldstein, D C Hellman & L D Peachey* The relation between the late after potential and the size of the transverse tubular system of frog muscle *J Gen Physiol* 1964 48 235
- Gastel M M & W Trøjnberg* Clinical and electrophysiological study of the pattern of conduction times in the distribution of the sciatic nerve *J Neurosurg Psychiat* 1964 27 351
- Gasser H S* Unmyelinated fibers originating in dorsal root ganglia *J Gen Physiol* 1950 33 651
- Gasser H S & J Erlanger* The nature of conduction of an impulse in the relatively refractory period *Amer J Physiol* 1925 73 613
- The role played by the sizes of the constituent fibers of a nerve trunk in determining the form of its action potential wave *Amer J Physiol* 1927 80 522
- The ending of the axon action potential and its relation to other events in nerve activity *Amer J Physiol* 1930 94 247
- Gerard R H & W H Marshall* Nerve conduction velocity and equilibration *Amer J Physiol* 1933 104 575
- Gillian R W & R G Willison* The refractory and supernormal periods of the human median nerve *J Neurol Neurosurg Psychiat* 1963 26 136
- Goodgold J & A Eberstein* Transmembrane potentials of human muscle cells in vivo *Exp Neurol* 1966 15 338
- Gottlieb T* The delay of the electrical response of nerve to a second stimulus *J Physiol (Lond)* 1910 40 250
- Graham H T* Supernormality: a modification of the recovery process in nerve *Amer J Physiol* 1934 110 225
- The subnormal period of nerve response *Amer J Physiol* 1935 111 452
- Graham H T & R Lorente de No* Recovery of blood perfused mammalian nerves *Amer J Physiol* 1938 123 326
- Grundfest H* Biochemical potentials *Ann Rev Physiol* 1940 2 213

- Håkansson C H* The propagation velocity of isolated frog muscle fibres *Acta physiol scand* 1954 31 suppl 114 22
- Conduction velocity and amplitude of the action potential as related to circumference in the isolated fibre of frog muscle *Acta physiol scand* 1956 37 14
- Håkansson C H* Action potentials recorded intra and extracellularly from the isolated frog muscle fibre in Ringer's solution and in air *Acta physiol scand* 1957 39 291
- Hermann L* Ueber den Actionsstrom der Muskeln im lebenden Menschen *Pflüger Arch ges Physiol* 1878 16 410
- den Hertog A* Responses of striated muscle fibers in vivo 1966 Theses Utrecht
- Hodder R* Linear relationship between fiber diameter and velocity of conduction in giant axon of squid *J Neurophysiol* 1953 16 145
- Hodgkin A L* The relation between conduction velocity and the electrical resistance outside a nerve fibre *J Physiol (Lond)* 1939 94 560
- Hodgkin A L & P Horowitz* The effect of sudden changes in ionic concentrations on the membrane potential of single muscle fibres *J Physiol (Lond)* 1960 153 370
- Hodgkin A L & A F Huxley* Potassium leakage from an active nerve fibre *J Physiol (Lond)* 1947 106 341
- Currents carried by sodium and potassium ions through the membrane of the giant axon of *loligo* *J Physiol (Lond)* 1952 116 449
- Hodgkin A L & B Katz* The effect of sodium ions on the electrical activity of the giant axon of the squid *J Physiol (Lond)* 1949a 108 37
- The effect of temperature on the electrical activity of the giant axon of the squid *J Physiol (Lond)* 1949b 109 240
- Isch F* *Electromyographie* 1963 Deren et C Paris
- Ishiko N & M Sato* The effect of calcium ions on electrical properties of striated muscle fibres *Jap J Physiol* 1957 7 51
- Jarcho L W, B Berman, R M Dowlen & J L Lilienthal Jr* Site of origin and velocity of conduction of fibrillary potentials in denervated skeletal muscle *Amer J Physiol* 1954 178 129
- Jenerick H P & R W Gerard* Membrane potential and threshold of single muscle fibers *J Cell Comp Physiol* 1953 42 79
- Johns H J* Microelectrode studies of muscle membrane potentials in man *Neuromuscular Disorders Res Publ Ass Nerv Ment Dis* 1960 38 704
- Katz B* The electrical properties of the muscle fibre membrane *Proc Roy Soc (London) ser B* 1948 135 506
- Keynes R D & P R Lewis* The sodium and potassium content of cephalopod nerve fibres *J Physiol (Lond)* 1951 114 151
- Krnjević K & R Miledi* Failure of neuromuscular propagation in rats *J Physiol (Lond)* 1958 140 440
- Kugelberg E* Accommodation in human nerves *Acta physiol scand* 1944 8 suppl 24
- Lorente de No R* A study of nerve physiology The Rockefeller institute for medical research New York 1947 131 132

- Lucas K. On the refractory period of muscle and nerve *J Physiol (Lond)* 1909 39 331
- Lucas K. The recovery of muscle and nerve after the passage of a propagated disturbance *J Physiol (Lond)* 1911 41 368
- Luttsau H C. The effect of metabolic inhibitors on the fatigue of the action potential in single muscle fibres *J Physiol (Lond)* 1964 178 45
- Macfarlane W V. Intracellular records of repetitive activity at the end plate of skeletal muscle fibres *J Physiol (Lond)* 1952 119 42
- Macfarlane W V & J D Meares. Modification of the after potential of single muscle fibres by 2,4-dinitrophenol *Nature* 1955 176 403
- Chemical modification of intracellularly recorded after potentials of frog skeletal muscle *J Physiol (Lond)* 1958a 142 78
- Intracellular recording of action and after potentials of frog muscle between 0 and 45°C *J Physiol (Lond)* 1958b 142 97
- Magladery J W, D B McDougal Jr & J Stoll. Electrophysiological studies of nerve and reflex activity in normal man *Bull Hopkins Hosp* 1950 86 313
- Meda E & A Ferroni. Sulla questione della velocità di conduzione dell'impulso nelle fibre muscolari striate dell'uomo *Arch Fisiol* 1957—58 57—58 76
- Moroney M J. Facts from figures. Penguin Books Ltd Harmondsworth Middlesex 1960
- van der Moss van Spijk D A, den Hertog & W Lammers. Intracellular negative stimulation of human muscle fibres *Proc 8th Congr of Neurol Vienna* 1965 p 417
- Noble D. Applications of Hodgkin-Huxley equations to excitable tissues *Physiol Rev* 1966 46 1
- Norris F H. Unstable membrane potential in human myotonic muscle *EEG Clin Neurophysiol* 1962 14 197
- Offner F A, Weinberg & G Young. Nerve conduction theory: Some mathematical consequences of Bernstein's model *Bull Math Biophys* 1940 2 89
- Persson A. The negative after potential of frog skeletal muscle fibres *Acta physiol scand* 1963 58 Suppl 205
- Pierce D S & I H Wagman. A method of recording from single muscle fibers or motor units in human skeletal muscle *J Appl Physiol* 1964 19 366
- Pillat B & P Heisracher. Veränderungen von Leitungsgeschwindigkeit und Latenz am Papilarmuskel der Katze während des Refraktärstadiums *Pflüger Arch* 1960 271 564
- Piper H. Über die Fortpflanzungsgeschwindigkeit der Kontraktionswelle im menschlichen Skelettmuskel *Z Biol* 1909 52 41
- Piper H. *Electrophysiologie menschlicher Muskeln*. Berlin: Verlag Julius Springer 1912
- Pond S E. Correlation of the propagation velocity of the contraction wave in muscle with the electrical conductivity of the surrounding medium *J Gen Physiol* 1921 3 807
- Pumphrey R J & J Z Young. The rates of conduction of nerve fibres of various diameters in cephalopods *J Exp Biol* 1938 15 453
- Riecker G H, D Bolte & M von Bubnoff. Ein Verfahren zur Messung von Einzelfaserpotentialen menschlicher Muskelzellen *in situ* 1963 *Pflüger Arch ges Physiol* 1963 277 231

- Schanne O R Kern & H Schafer* Zum Problem des Innenwiderstandes biologischer Zellen *Naturwissenschaften* 1962 49 161
- Seno S* A study of synchronous activity of paretic muscles in human poliomyelitis by means of vector electromyogram *Tohoku J Exper Med* 1961 75 55
- Snedecor G W* Statistical Methods 1956 5th Iowa State College Press Ames Iowa
- Stålberg E & J Ekstedt* Propagation velocity in voluntarily activated human muscle *Electroenceph Clin Neurophysiol* 1965 18 211
- Stålberg E L O Dahlback & J Ekstedt* Ischemiska effekter på den neuro-muskulära transmissionen och på propagationshastigheten i muskelfibrer *Nord Med* 1966 75 764
- Standaert F G* Post tetanic repetitive activity in the cat soleus nerve *J Gen Physiol* 1963 47 53
- Tasaki I* The excitatory and recovery process in the fibre is modified by temperature changes *Biochim Biophys Acta* 1949 3 498
- Ussing H H* Ionic movements in cell membranes in relation to the activity of the nervous system *Ergebn Physiol* 1959 50 159
- Wilska A & K Varjaranta* Über die Temperaturabhängigkeit der Leitungsgeschwindigkeit des Aktionspotentials einer Muskelfaser *Skand Arch Physiol* 1940 83 88







ACTA PHYSIOLOGICA SCANDINAVICA

Vol 70 Supplementum 288

THE DISTRIBUTION OF  
ACETYLCHOLINE ESTERASE AND  
OTHER HYDROLYTIC ENZYMES  
IN RETINAL CULTURES

By

HANS ARNE HANSSON

GÖTEBORG 1966



ACTA PHYSIOLOGICA SCANDINAVICA

Vol 70 Supplementum 288

---

The Institute of Neurobiology and the Neuropathological Laboratory  
Institute of Pathology I University of Göteborg Göteborg Sweden

THE DISTRIBUTION OF  
ACETYLCHOLINE ESTERASE AND  
OTHER HYDROLYTIC ENZYMES  
IN RETINAL CULTURES

By

HANS ARNE HANSSON

GÖTEBORG 1966



In the retina high AChE<sup>+</sup> activity has been demonstrated in the inner plexiform layer and in the ganglion cell layer but no or very low in the outer nuclear layer (for review see Eichner 1958 Esila 1963, Koelle 1963 Rohen 1964). The AChE activity increases during differentiation *in vivo* of the nerve cells (for discussion see Karczmar 1963 Couteaux 1963). After damage of nerve structures the AChE activity soon reaches its normal level after an initial decrease (for discussion see Fredricsson and Sjoqvist 1962 Harkonen 1964).

Recent electron microscopic and electrophysiological studies on cultured nervous tissue have demonstrated synapses in the explants (Bunge Bunge and Peterson 1964 Crain and Bornstein 1964 Callas and Hild 1964 Crain and Peterson 1964 Peterson Crain and Murray 1965, Murray 1965). Except for the studies of Geiger and Stone (1962) which have recently been criticized (Bornstein 1964 Murray 1965) no other histochemical study of AChE activity in cultures of nervous tissue has been published.

Previous studies on retinal cultures have demonstrated that the nerve cells develop with time *in vitro* (Hansson and Sourander 1964 Hansson 1965 1966). This study was performed to investigate if AChE occurs in the nerve cells including their processes *in vitro*. As there are many histochemical methods for AChE and no reliable studies on AChE activity on cultures of nervous tissue have been published several methods were tested in this study combined with inhibitors with known properties.

The chemical mediator for transmission of impulses from the visual cells to the nerve cells in the inner nuclear layer is unknown *in vivo* (for discussion see Esila 1963 Rohen 1964). Therefore also the visual cells in the explants were examined for possible AChE activity.

The nonspecific esterases and other hydrolytic enzymes in the mature retina *in vivo* have a characteristic distribution with especially high activities in the visual cells (for review see Eichner 1958, Esila 1963 Rohen 1964). Therefore the retinal cultures and particularly the photoreceptor cells in the retinal cultures were investigated histochemically for such enzymes as they might indicate a cellular development.

Abbreviations used: AChE = Acetyl choline esterase ChE = Choline esterase ns E = Nonspecific esterases F = Esterase

## MATERIAL AND METHODS

*Experimental material* Most of the experiment were carried out on retinal tissue from one to two day old Sprague-Dawley rats and albino rabbits. In addition cultures were also prepared from embryonic rats newborn and young rats of up to four weeks age, and from young cats and mice. In all about 3000 selected growing cultures were used in this study.

*Culture technique* The original tissue culture method and its later modifications have previously been described in detail (Hansson and Sourander 1964, Hansson 1965, 1966). Briefly, small pieces of retinal tissue varying in size from a few cells up to about a cubic millimeter were explanted directly onto clean cover glasses without any collagen or plasma clot. One of the problems was to ensure that the connections between the different neuroectodermal cells and their processes were formed in culture and not surviving from the organization existing in the retina at the time of explantation. Therefore the tissue pieces were dispersed mechanically into clumps of up to ten cells by being drawn repeatedly into pipettes prior to the explantation. Larger tissue pieces were usually used for histochemical studies. The explants were grown in Leighton tubes or in Gey chambers. The nutrient medium consisted of a modified Hank's solution with high glucose concentration (500—600 mg per 100 ml) and 20% calf serum with addition of penicillin and streptomycin.

### General appearance of the retinal cultures

The appearance of the retinal cultures has previously been described (Hansson and Sourander 1964). Briefly, clusters of cells grow on the surface of a monolayer of large, thin polygonal cells. These latter cells which form argentophilic precollagen networks, are considered to be derived chiefly from the mesenchymal cells in the blood vessels in the retina.

A few of the cells on the surface of the monolayer have a phase dense cytoplasm and several long branching processes. The phase light homogeneous nucleoplasm in the spherical nucleus contains a single nucleolus. Their perikaryon contain neurofibrils and Nissl substance. Their size increases and the Nissl substance aggregates and stains more intensely during the first two weeks in vitro. They are interpreted as nerve cells from the ganglion cell layer and to a certain extent also from the inner nuclear

layer of the retina. Small cells of uniform size, about 7-9  $\mu$  in diameter, with polar processes. Nissl substance and neurofibrils in their scanty cytoplasm and with a phase light spherical or kidney shaped nucleus stain intensely with the drug M 223 when observed in a fluorescence microscope. They correspond to the bipolar nerve cells in the inner nuclear layer of the intact retina.

The majority of the cells are small and of uniform size. The scanty cytoplasm, just forming a thin rim around the nucleus, seems to lack neurofibrils and Nissl substance. A straight process of uniform thickness and a very thin branching one emerge from opposite poles. The nucleus is filled with two to six large chromatin conglomerates. These cells form rosettes with a central lumen, into which the thicker processes of the cells protrude. The lumen is bordered by a membranous structure. These cells are interpreted as visual cells.

On the surface of the monolayer there are multipolar cells with a slightly granulated cell body. The oval single nucleus is difficult to observe because of the small difference in the phase density between the nucleoplasm and the cytoplasm. They lack Nissl substance. Their processes often show webbing at the points of branching. These cells are interpreted as neuroglial cells.

The processes from the different neuroectodermal cells are in connection with each other and with cell bodies. New connections are continuously established while others are broken with time in culture. Most of them are of the types end to side or end to end. Processes from the same type of cell or neuroglial cell are often observed to establish a complex system of connections. Nerve bundles are exceptional in the retinal culture.

A varying number of phagocytes are observed among the cultured cells in the explants. Some of the large granular cells phagocytose dead cells on the surface of the monolayer or among the cells in the explant. They are stained with Azur B and resazurin. They are of the type of macrophages. The small round cells are also phagocytic. They are of the type of microphages. They are also stained with Azur B and resazurin. They are also of the type of macrophages.

#### Phagocytic cells in culture

During the first 24 hours of culture, the phagocytic cells are of the type of macrophages. They are large and granular. They are stained with Azur B and resazurin. They are also of the type of microphages. They are small and round. They are also stained with Azur B and resazurin. They are also of the type of macrophages.



inhibitors described below were usually added to the nutrient medium half an hour before the slides were transferred to the incubation solution. However, in six experimental series the explants were preincubated with the inhibitors in Holmstedt's solution without substrates for half an hour as described by Holmstedt (1957). This was done in order to compare the results obtained on living and on dead cells. Most of the explants were unfixed but a few were fixed in ice cold formalin calcium. The incubation times were 30 minutes to four hours at 37° C. Marked diffusion artefacts were evident only after prolonged incubation of about 12 hours.

Gomori's modification (1952) of Koelle's thiocholine method was also used for demonstration of AChE in both formalin fixed and unfixed explants in five experiments.

The  $\alpha$  naphthyl acetate method of Nachlas and Seligman was used as described by Barka and Anderson (1963). The substrate used was  $\alpha$  naphthyl acetate (Sigma) with Fast Blue RR salt (Sigma) as coupling agent. The incubation time at 37° C was 3—5 minutes for unfixed cultures and 30 minutes for those fixed for 5 minutes in ice cold formalin.

A few experiments were performed according to the naphthol AS D acetate method (Barka and Anderson 1963).

Holt's method for substituted indoxyl acetates was also used with  $\alpha$  acetyl 5 bromoindoxyl and  $o$  acetyl 4 chloro 5 bromoindoxyl (Sigma) as substrates (Barka and Anderson 1963).

Parallel controls were run in incubation solutions without the substrates or coupler and on cultures which had been immersed in 65° C water for 5 minutes. The controls were always unstained.

*Inhibitors* Eserine salicylate (E. Merck A.G., Darmstadt, Germany)  $1 \times 10^{-7}$  M was used for inhibition of ChE. AChE was inhibited by BW 284 C 51 (1,5 bis(N allyl N,N dimethyl 4 ammoniumphenyl)pentan-3-one dibromide, Wellcome Research Laboratories, Beckenham, England) at  $1 \times 10^{-6}$  M and ns ChE by  $1 \times 10^{-5}$  M IsoOMPA (tetramonoisopropyl pyrophosphortetramide, Light)  $4 \times 10^{-6}$  M Mipafox (N,N diisopropyl phosphorodiamidic fluoride, Light) or in a few experiments with Holmstedt's method  $3 \times 10^{-8}$  M Nu 683 (dimethylcarbamate of 1,2 hydroxy 5 phenylbenzyltrimethylammonium bromide, Hoffman-La Roche, Basel, Switzerland). E 600 (diethyl 4 nitrophenyl phosphate, Mintacol soluble, Bayer, Leverkusen, Germany) was used at a concentration of  $1 \times 10^{-5}$  M.

*Demonstration of acid phosphatases* The histochemical demonstration of acid phosphatases was performed both on fixed and on unfixed retinal cultures according to the methods of Gomori and of Burstone (for technical details see Pearse 1960, Barka and Anderson 1963).

*Demonstration of leucyl naphthyl amide hydrolase* The presence of this enzyme was shown as described by Nachlas *et al* using L-leucyl  $\beta$  naphthyl amide hydrochloride or L leucyl-4 methoxy  $\beta$  naphthyl amide hydrochloride (Sigma) as substrates (for technical details see Barka and Anderson 1963)

*Demonstration of nucleotidases and pyrophosphatase* Dinucleotidases were demonstrated according to the method of Novikoff and Goldfischer (1961) using adenosine diphosphate, inosine diphosphate and uridine diphosphate (Sigma) as substrates. Pyrophosphatase was demonstrated with the same method using tiamine pyrophosphate (Sigma) as substrate. Adenosine triphosphatase was shown according to the method of Wachstein and Meisel (Barka and Anderson 1963) using adenosine triphosphate (Sigma) as substrate.

#### Evaluation of the histochemical reactions obtained with the methods for esterases

The enzyme activity demonstrated with acetylthiocholine iodide as substrate and with either of the inhibitors IsoOMPA, Mipafox or Nu 683 in the incubation solution, was interpreted as depending upon AChE (for discussion see Holmstedt 1957, Pearse 1960, Holmstedt and Sjoqvist 1961, Barka and Anderson 1963, Koelle 1963, Digenbach 1965). It was entirely inhibited by BW 284 C 51 (Table 1). Activity resistant to IsoOMPA or Mipafox but sensitive to BW 284 C 51 obtained with the other used esterase substrates was supposed to reflect AChE. Ns ChE activity was demonstrated with butyrylthiocholine iodide as substrate and BW 284 C 51 as inhibitor of AChE. It was inhibited with IsoOMPA and Mipafox (Table 1). The ChE activity was inhibited by eserine. The eserine resistant ns E activity was further studied with E 600.

The unfixed retinal cultures showed the highest activity as shown with the different histochemical methods as compared to formalin fixed explants. However the latter showed the best preservation of the cellular structures. In no case were the signs of diffusion marked until after prolonged incubation.

inhibitors described below were usually added to the nutrient medium half an hour before the slides were transferred to the incubation solution. However in six experimental series the explants were preincubated with the inhibitors in Holmstedt's solution without substrates for half an hour as described by Holmstedt (1957). This was done in order to compare the results obtained on living and on dead cells. Most of the explants were unfixed but a few were fixed in ice cold formalin calcium. The incubation times were 30 minutes to four hours at 37° C. Marked diffusion artefacts were evident only after prolonged incubation of about 12 hours.

Gomori's modification (1952) of Koelle's thiocholine method was also used for demonstration of AChE in both formalin fixed and unfixed explants in five experiments.

The  $\alpha$  naphthyl acetate method of Nachlas and Seligman was used as described by Barka and Anderson (1963). The substrate used was  $\alpha$  naphthyl acetate (Sigma) with Fast Blue RR salt (Sigma) as coupling agent. The incubation time at 37° C was 3—5 minutes for unfixed cultures and 30 minutes for those fixed for 5 minutes in ice cold formalin.

A few experiments were performed according to the naphthol AS D acetate method (Barka and Anderson 1963).

Holt's method for substituted indoxyl acetates was also used with o acetyl 5 bromoindoxyl and o acetyl 4 chloro 5 bromoindoxyl (Sigma) as substrates (Barka and Anderson 1963).

Parallel controls were run in incubation solutions without the substrates or coupler and on cultures which had been immersed in 65° C water for 5 minutes. The controls were always unstained.

*Inhibitors* Estrine silicylate (E. Merck A. G., Darmstadt, Germany)  $1 \times 10^{-5}$  M was used for inhibition of ChE. AChE was inhibited by BW 284 C 51 (1,5 bis(N allyl)-N,N dimethyl 4 ammoniumphenyl/pentan-3-one dibromide, Wellcome Research Laboratories, Beckenham, England) at  $1 \times 10^{-5}$  M and not ChE by  $1 \times 10^{-5}$  M IsoOMPA (tetramonoisopropyl pyrophosphortetramide, Light),  $4 \times 10^{-6}$  M Mipafox (N,N-diisopropyl phosphorodiamidic fluoride, Light) or in a few experiments with Holmstedt's method  $3 \times 10^{-8}$  M Nu 683 (dimethylcarbamate of 1/2 hydroxy 5 phenylbenzyl/trimethylammonium bromide, Hoffman La Roche, Basel, Switzerland). E 600 (diethyl 4 nitrophenyl phosphite, Mintacol soluble, Bayer, Leverkusen, Germany) was used at a concentration of  $1 \times 10^{-5}$  M.

*Demonstration of acid phosphatases* The histochemical demonstration of acid phosphatases was performed both on fixed and on unfixed retinal cultures according to the methods of Gomori and of Burstone (for technical details see Pearse 1960, Barka and Anderson 1963).

## RESULTS

### Esterase methods

**Thiocholine methods** The method used in most cases for demonstration of AChE activity in nerve cells in the retinal cultures of different origins was that of Holmstedt (1957) slightly modified for the tissue culture conditions. The copper thiocholine complex formed (Malmgren and Sylven 1955) was precipitated as small distinct granules on the surface of the nerve cell body in the cytoplasm especially in the vicinity of the cell membrane and on its processes. It was often possible to observe a single process for a long distance with the precipitate forming a string of pearls on it. The discontinuous staining sometimes observed did not correspond to any visible break of the processes as observed in phase contrast microscopy. Processes from different nerve cells with signs of AChE activity were observed in close connection with each other. Most of the multipolar nerve cells were stained. Among those which failed to stain a few showed signs of degeneration. The intensity of the staining varied both between different cultures and in the same explant. A number of small polar nerve cells also showed precipitates indicating AChE activity especially when observed in a phase contrast microscope (Table 2).

Table 2

Distribution of esterase activity in unfixed rabbit retinal cultures with acetylthiocholine iodide as substrate (Holmstedt's method)

Inhibitor	Multipolar nerve cells		Small nerve cells	Neuroglial cells	Rod cells		Mono-layer cells	Granulated mesenchymal cells
	Cell body	Processes			Cell body	Inner segm.		
No inhibitor	+	++	(+)	—	—	—	—	(+)
Mipafox	+	++	(+)	—	—	—	—	(+)
IsoOMPA	+	++	(+)	—	—	—	—	(+)
BW 284 C 51	—	—	—	—	—	—	—	(+)
IsoOMPA & BW 284 C 51	—	—	—	—	—	—	—	(+)
Eserine	—	—	—	—	—	—	—	(+)
E 600	—	—	—	—	—	—	—	(+)

+++ = Strong activity  
 ++ = Moderate activity  
 + = Light activity

— = No activity  
 ( ) = Activity only in some of the cells

Table 1

The effects of the inhibitors on cholinesterases

Substrate	ChE hydrolyzing		Inhibitor	ChE inhibited		ChE demonstrated	
	AChE	ns ChE		AChE	ns ChE	AChE	ns ChE
AcThCh	+	+	No inhibitor	—	—	+	+
	+	+	Mipafox	—	+	+	—
	+	+	IsoOMPA	—	+	+	—
	+	+	Nu 683	—	+	+	—
	+	+	BW 284 C 51	+	—	—	+
	+	+	IsoOMPA & BW 284 C 51	+	+	—	—
	+	+	Eserine	+	+	—	—
	+	+	E 600	+	+	—	—
BuThCh	—	+	No inhibitor	—	—	—	+
	—	+	Mipafox	—	+	—	—
	—	+	IsoOMPA	—	+	—	—
	—	+	Nu 683	—	+	—	—
	—	+	BW 284 C 51	+	—	—	+
	—	+	IsoOMPA & BW 284 C 51	+	+	—	—
	—	+	Eserine	+	+	—	—
	—	+	E 600	+	+	—	—

Abbreviations AcThCh = Acetylthiocholine iodide  
 BuThCh = Butyrylthiocholine iodide

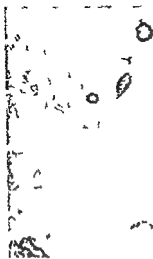


Fig 3 Acetylcholine esterase activity in a nerve cell after 15 days in culture. Retinal explant from a 1 day old albino rabbit. Stained according to Holmstedt with  $1 \times 10^{-4}$  M IsoOMPA as inhibitor. Note the stained granular mesenchymal cell in the lower left corner of the figure. Incubated for 4 hours at  $37^{\circ}$  unfixed.



Fig 4 Acetylcholine esterase activity in a retinal nerve cell after 18 days in vitro from a 2 day old albino rabbit. Stained according to Holmstedt using  $3 \times 10^{-3}$  M Nu 683 as inhibitor. Incubated unfixed for 3 hours.



Fig 5 Phase contrast microscopy picture of the nerve cell in Fig 2 showing the close relation between the processes and the pre-empt.



Fig 6 Nerve cells (arrows) showing acetylcholine esterase activity in a 15 day old retinal culture from a 2 day old albino rabbit. Stained according to Gomori using  $1 \times 10^{-3}$  M IsoOMPA as inhibitor. Incubated for 4 hours after 10 minutes fixation in cold formalin calcium.



Fig 7 Retinal culture after 14 days in vitro from a 2 day old Sprague Dowley rat. Stained for acetylcholine esterase activity according to Holmstedt using  $1 \times 10^{-6}$  M IsoOMPA as inhibitor. Note the staining of the nerve cells and their processes and the unstained visual cells (arrows). Incubated unfixed for 1 hour.

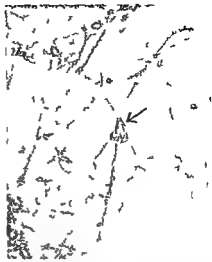


Fig 8 In the centre of the field a large multipolar nerve cell (arrow) stained for acetylcholine esterase activity according to Holmstedt using  $4 \times 10^{-6}$  M Mipafox as inhibitor. Fixed in cold formalin calcium for 10 minutes and incubated for 4 hours. The nerve cell processes show staining.

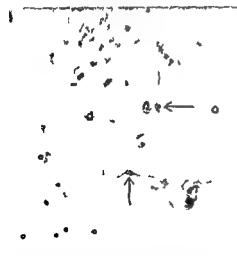


Fig 9 Nerve cells (arrows) in a retinal culture stained for acetylcholine esterase activity according to Holmstedt using  $4 \times 10^{-6}$  M Mipafox as inhibitor. Unfixed 25 day old retinal culture from a 2 day old Sprague Dowley rat. Note the stained granulated mesenchymal cells in the lower part of the picture. Brightfield.



Fig 10 Phase contrast microscopy picture of the same field as in Fig 9 showing the nerve cell processes.

There was no significant difference in the effects of the inhibitors or in the distribution of the precipitate between those cultures preincubated with the inhibitors in the nutrient medium and those which were preincubated in Holmstedt's solution without substrate

The neuroglial cells and the visual cells remained unstained as well as the cells of the monolayer. However, a few of the granulated mesenchymal cells showed a slight AChE activity as previously defined (Table 1) in their cytoplasmic granules, sometimes possible to observe only in phase contrast microscopy (Figs 3, 9 and 10). Such precipitates were also seen in cultures treated with BW 284 C 51 with IsoOMPA and BW 284 C 51 together and with eserine. These pretreatments as well as E 600 inhibited all activity in the nerve cells (Table 2).

No ChE activity with butyrylthiocholine iodide as substrate and BW 284 C 51 as inhibitor of AChE was observed in the granulated mesenchymal cells. A very slight activity could be seen in some of the larger nerve cells and in scattered neuroglial cells, especially in old cultures after prolonged incubation.

The thiocholine method of Gomori revealed AChE activity in the perikaryon but not in the processes of the nerve cells. The precipitated amorphous material was of varying size and less distinct than that with the method of Holmstedt (Fig. 6).

AChE activity was not observed in the nerve cells during the first week in culture. The number and the size of the precipitates increased during the next week and did not change significantly thereafter. This was true both for explants derived from large retinal tissue pieces and for those formed by aggregation of mechanically dispersed cells. AChE activity was demonstrated in nerve cells in cultures after up to 2½ months in vitro, i.e. the longest time examined. No nuclear staining was observed.

*a Naphthyl acetate method* The results obtained are summarized in table 3. There was no difference between the results obtained on cultured nerve cells without any inhibitor, with the inhibitors IsoOMPA and BW 284 C 51 alone or together. Eserine and E 600 reduced the staining intensity.

The different types of nerve cells showed a moderate n.s.E. activity. However, the largest multipolar nerve cells and scattered small nerve cells were heavily stained. The brown precipitate was evenly distributed throughout the cytoplasm and the largest processes. A weak reaction was observed in the neuroglial cells. The cell body in the photoreceptor cells was unstained or revealed a very weak E. activity while the proximal parts of the rod-like processes corresponding to the inner segments were heavily stained.



Table 3

Distribution of esterase activity in unfixed rabbit retinal cultures with  $\alpha$  naphthyl acetate as substrate

Inhibitor	Multipolar nerve cells		Small nerve cells	Neuroglial cells	Rod cells		Mono-layer cells	Granulated mesenchymal cells
	Cell body	Processes			Cell body	Inner segm		
No inhibitor	++(+)	++	+	(+)	+	+++	+	+++
IsoOMPA	++(+)	++	+	(+)	+	+++	+	+++
BW 284 C 51	++(+)	++	+	—	+	+++	+	+++
IsoOMPA & BW 284 C 51	++(+)	+	+	—	+	+++	+	+++
Eserine	+(+)	+	(+)	—	+	++(+)	+	+++
E 600	+	—	—	—	—	+(+)	+	++

The granulated mesenchymal cells showed high E activity in their cytoplasmic granules

The relative E activity of the cell types and the dye distribution were roughly the same in unfixed and in formalin fixed cultures although the incubation time was longer in the latter case

Table 4

Distribution of esterase activity in unfixed rabbit retinal cultures with naphthol AS D acetate as substrate

Inhibitor	Multipolar nerve cell		Small nerve cells	Neuroglial cells	Rod cells		Mono layer cells	Granulated mesenchymal cells
	Cell body	Processes			Cell body	Inner segm		
No inhibitor	+(+)	(+)	(+)	—	(+)	+++	—	+++
IsoOMPA	+(+)	(+)	—	—	—	+++	—	+++
BW 284 C 51	+(+)	(+)	—	—	—	+++	—	+++
IsoOMPA & BW 284 C 51	+(+)	(+)	—	—	—	+++	—	+++
Eserine	(+)	—	—	—	—	(+)	—	+(+)
E 600	(+)	(+)	—	—	—	+(+)	—	++



Fig 11 Incubated without any inhibitor

Fig 11--13 Unfixed retinal cultures after 14 days in vitro from a 2 day old albino rabbit Stained for esterase activity according to Holt using *o* acetyl 4 chloro 5 bromo indoxyl as substrate

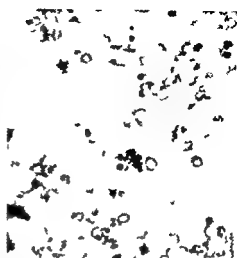


Fig 12 Incubated with  $1 \times 10^{-5}$  M IsoOMPA

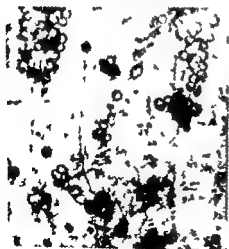


Fig 13 Incubated with  $1 \times 10^{-5}$  M BW 284 C 51

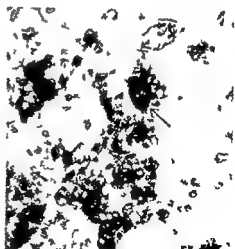


Fig 14 Unfixed retinal culture from a 2 day old Sprague Dowley rat after 16 days in vitro Stained for esterase activity using *o* naphthyl acetate as substrate and  $1 \times 10^{-5}$  M IsoOMPA as inhibitor All the neuroectodermal cells are stained as well as a granulated mesenchymal cell (arrow)

The staining pattern described in table 3 did not change with the age of the explants in vitro although the esterase activity increased during the first three or four days in culture

*Naphthol AS-D acetate method* The naphthol AS D acetate method gave distinct coarse and irregular green granules They were observed in the cytoplasm often in the vicinity of the cell membrane The large nerve cell processes showed irregularly distributed, fine granules No nuclear activity was observed The distribution is described in table 4

*Indoxyl acetate method* Large, green crystalloid granules were obtained when o acetyl 5-bromoindoxyl was used as substrate In contrast ■ acetyl-4 chloro 5 bromoindoxyl as substrate gave a fine grained distinct green staining without any marked tendency for formation of large amorphous precipitate

Formalin fixation reduced markedly the rate of hydrolysis without any definite change in the localization or relative intensity of the staining (Table 5 Figs 11—13, 17, 18)

Table 5

Distribution of esterase activity in unfixed rabbit retinal cultures with o acetyl 4 chloro 5 bromoindoxyl as substrate

Inhibitor	Multipolar nerve cells		Small nerve cells	Neuroglial cells	Rod cells		Mono-layer cells	Granulated mesenchymal cells
	Cell body	Processes			Cell body	Inner segm		
No inhibitor	+++	+	++	—	(+)	+++	—	++
IsoOMPA	+(++)	+	+	—	—	+++	—	+++
BW 284 C 51	+(++)		+	—	—	+++	—	+++
IsoOMPA & BW 284 C 51	+(++)	+	+	—	—	+	—	+++
Eserine	+(+)	—	—	—	—	++	—	+++
E 600	(+)	—	—	—	—	++	—	++

It seems likely that 4 chloro 5 bromoindoxyl acetate was hydrolysed by enzymes corresponding to AChE because the staining intensity was reduced from strong to moderate by incubation with BW 284 C 51 in many but not all of the multipolar nerve cells This difference was especially pronounced in explants incubated only half of the normal time period



Fig 15 Retinal culture stained for esterase using *n* naphthyl acetate as substrate and eserine as inhibitor. Formalin fixed for 5 minutes prior to the incubation. Note the high activity in the rosettes (arrows). 12 day old culture from a 2 day old Sprague Dowley rat



Fig 16 Retinal culture stained as in Fig 15 but with E 600 as inhibitor. Formalin fixed



Fig 17 Explant of retinal tissue from a 2 day old Sprague Dowley rat after 10 days in vitro stained with naphthol ASD acetate as substrate and with IsoOMPA and BW 284 C 51 in combination as inhibitor. Note the strong intensity of the staining in the rosettes



Fig 18 Heavily stained granulated mesenchymal cells on the surface of a monolayer of mesenchymal cells. Phase contrast microscopy of a formalin fixed 21 day old retinal culture from a 2 day old Sprague Dowley rat. o Acetyl 4 chloro 5 bromo-indoxyl in combination with eserine

## Acid phosphatases

The method of Burstone using naphthol AS TR phosphate AS BI phosphate and AS MX phosphate as substrates and that of Gomori using  $\beta$  glycerophosphate as substrate gave results in agreement with each other. Details in table 6

Table 6

Histochemical activity of acid phosphatase (APhs) leucyl naphthyl amide hydrolase (LNAs) uridine dinucleotidase (UDPs) and adenosine triphosphatase (ATPs) in formalin fixed rabbit retinal cultures

Enzyme	Multipolar nerve cells		Small nerve cells	Neuroglial cells	Rod cells		Mono-layer cells	Granulated mesenchymal cells
	Cell body	Processes			Cell body	Inner segm		
APhs	++(+)	+	+	+	+	+++	+	+++
LNAs	+(+)	—	+	(+)	—	++(+)	—	++(+)
UDPs	++	—	+	—	+	+++	+	+++
ATPs	++(+)	—	++	—	+	+++	(+)	+++

## Leucyl naphthyl amide hydrolase

The distribution of leucyl naphthyl amide hydrolase was similar to that of acid phosphatases in its principle cellular distribution pattern (Table 6). The dye deposits were mostly finely granular but some diffusely distributed stain was also evident.

## Dinucleotidases and pyrophosphatase

The distribution of dinucleotidases is shown in table 6. There were no significant differences between the histochemical results with the substrates used i.e. uridine 5 diphosphate, inosine 5 diphosphate and adenosine 5 diphosphate. The intensity of the staining increased during the first two weeks in vitro. Similar results were obtained when using thiamine pyrophosphate as substrate. However the results with the latter substrate were less reproducible and often showed marked variations in the intensity of the staining. The formed precipitate was less distinct with thiamine pyrophosphate than with the dinucleotides.

The precipitates were fine grained after the histochemical staining for dinucleotidases. Large distinct thread like deposits in the perinuclear region of the multipolar nerve cells were rarely evident and only after at least six weeks in culture in the largest nerve cells. The cell membrane of the granulated mesenchymal cells were distinctly outlined after staining for dinucleotidases making it very easy to observe the complex pattern of thin expansions from these cells.

#### Adenosine triphosphatase

The obtained results were similar to those for dinucleotidases (Table 6). No distinct perinuclear staining in the nerve cells was observed.

## DISCUSSION

The principal aim of this study was to investigate if AChE is present in the nerve cells including their processes in the retinal cultures. Since nothing is known about the reliability of the histochemical methods for AChE when used on cultured nervous tissue, several esterase methods were used and the results compared with each other.

For histochemical studies on retinal tissue from living animals it is necessary either to freeze and section or to fix, embed and section the tissue to be examined. However, in the cultures the cells with their membranes intact and the organelles undamaged may be immersed into the incubation solution. Both unfixed and formalin fixed explants were examined histochemically in the present study for comparison of the effects of these different pretreatments. Formalin fixation reduced the esterase activity in the cells in the explants but neither the distribution of the dye precipitates in the cells including the processes nor the relative intensity of the staining of the different cell types did seem to be significantly changed by these two different pretreatments.

A further source of error to be avoided was the occurrence of diffusion artefacts. The importance of including sodium sulphate in amounts large enough to minimize the diffusion of the enzyme and of the reaction products formed in the cells during the incubation has been stressed in the literature (Gomori 1952, Holmstedt 1957, Koelle 1963). With the method of Holmstedt precipitates of copper thiocholine complex were formed in the cytoplasm especially in the vicinity of the cell membrane and along the nerve cell processes. They appeared as distinct granules. Careful examinations with phase contrast microscopy failed to reveal any diffusely located precipitate or precipitate not related to nerve cell structures. Similar results were obtained with the thiocholine method of Gomori, although fine amorphous material was observed not only in the nerve cells but also outside the cell body just around the nerve cells. There was no certain difference between unfixed and formalin fixed explants. An important difference between the two thiocholine methods was that no precipitates were observed along the nerve cell processes after staining according to Gomori for AChE. No nuclear activity was seen with either of these two thiocholine methods.

AChE activity could be demonstrated in the nerve cells only with the

two thiocholine methods. The most reproducible results were obtained with Holmstedt's modification of Koelle's method. The precipitates of the copper thiocholine complex (Malmgren and Sylven 1955) were formed as distinct granules which were localized both to the nerve cell cytoplasm and to the processes but never to the nucleus. Giacobini (1956) has demonstrated the same distribution of AChE activity in freshly isolated unfixed nerve cells of the central and the peripheral nervous system. The results of the staining for AChE activity in the perikaryon of nerve cells including the processes are also in agreement with those for nervous tissue *in vivo* (Holmstedt 1957, Holmstedt and Sjöqvist 1961, Couteaux 1963, Koelle 1963, Ehinger 1966). The AChE activity in the cultured nerve cells was resistant to Mipafox, IsoOMPA and Nu 683 but was completely inhibited by BW 284 C 51, eserine and E 600 (Table 2).

AChE activity was also demonstrated in the cultured retinal nerve cells after staining according to the method of Gomori. However, only the perikaryon and not the processes showed precipitates (Fig. 6).

Indoxyl acetate seemed to be the only of the other substrates which at least partly was hydrolysed by AChE. The inhibitor BW 284 C 51 caused a slight decrease in the activity in the cytoplasm and the processes of most of the nerve cells. However, the nerve cells showed moderate esterase activity even after treatment with IsoOMPA and BW 284 C 51 in combination which made the results unreliable (Table 3).

Thus, Holmstedt's modification of Koelle's thiocholine method adapted to the tissue culture conditions was found to be suitable for demonstration of AChE activity in nerve cells in tissue cultures.

In the retinal cultures AChE activity was demonstrated in the multipolar nerve cells and in some of the small nerve cells. The visual cells as well as the neuroglial cells lacked AChE activity. In the retina of rats, rabbits, mice and cats AChE activity occurs *in vivo* in the inner plexiform layer and in the ganglion cell layer (Enslin 1963, Koelle 1963, Ehinger 1966). High activity of AChE has been demonstrated in the anterior colliculi, especially in the layers of the tectum where the optic fibres terminate (for discussion see Couteaux 1963). Thus, the results obtained on the retinal cultures correspond with those described to occur *in vivo*.

Connections between different nerve cell processes of the types end to end and end to side between crossing processes and between nerve cell processes and perikaryon were often observed both in living cultures with phase contrast microscopy and in fixed and stained cultures. The silver staining method of Holmes was observed to be suitable for observations and analysis of the complicated network of dendrites and axons in the



tivity *in vitro* was partly resistant to pretreatment with the potent inhibitor E 600 both in unfixed and in formalin fixed retinal explants. The latter enzyme activity may indicate the presence of peptidases (Pearse 1960). Previously, the same localization of enzyme activity has been shown for pyrophosphatase, dinucleotidases and adenosine triphosphatase (Hansson 1966). The activity of these hydrolysing enzymes increased during the first days in culture.

It is tempting to speculate about the cellular structures which show these high enzyme activities. The lysosomes have been stated by definition to contain acid phosphatases, peptidase and esterases (Novikoff 1963). Lysosome like structures have been described in the inner segments of the visual cells *in vivo* (Rohen 1964, Sjostrand and Nilsson 1965). The pyrophosphatase and dinucleotidases have been localized to the Golgi complex (Novikoff and Goldfischer 1961, Torack and Barnett 1963, Allen 1963, Shanthaveerappa and Bourne 1965). Nucleoside triphosphatase activity including adenosine triphosphatase activity has been demonstrated in the outer and inner segments of the visual cells as well as in the plexiform layers (Scarpelli and Craig 1963). Thus the high enzyme activity of the proximal part of the rod-like processes of the photoreceptor cells shown in this study may be due to the presence of lysosomes and other cytoplasmic structures with high enzyme activity. The histochemical results demonstrate that the enzymatic activity of these structures increases during the first time *in vitro*, in the same way as in the intact retina *in vivo* indicating a chemical differentiation.

High activities of ns E and E 600 resistant E were observed in the proximal part of the rod like processes of the photoreceptor cells both in fixed and in unfixed retinal explants. These results are not in agreement with those of Esila (1963) who could demonstrate ns E *in vivo* only in formalin fixed nerve cells but not in unfixed. In her study no E 600 resistant E activity could be shown in the inner segments of the visual cells. These contradictory results may be due to differences in the treatment of the nervous tissue. Esila used cryostat microtome sections which were allowed to dry prior to the incubation. In the present study the retinal cultures were placed directly into the substrate solution without any fixation, freezing, sectioning or drying of the tissue.

Histochemical studies on the retinal cultures for pyrophosphatase and dinucleotidases revealed precipitates diffusely distributed in the perikaryon of the nerve cells. The deposits were fine grained. Large threadlike precipitates like those observed *in vivo* (Shanthaveerappa and Bourne 1965) were rarely seen in the cultured nerve cells. They could occasionally be

recognized after several weeks in vitro. In light and in electron microscopic cytochemical studies the pyrophosphatase and the dinucleotidase activities have been localized to the Golgi complex (Novikoff and Goldfischer 1961, Torack and Barnett 1963, Allen 1963). Previous studies on cultured nerve cells have failed to demonstrate any lamellar network after histological staining resembling the Golgi apparatus (for review see Hild 1959). Studies in vivo on regenerating nerve cells in the central nervous system revealed an initial decrease in the activity of dinucleotidases and pyrophosphatase and that the lamellar structures of the Golgi complex grew thinner (Barron and Tuncbay 1964, Sjostrand 1966). The cellular conditions prevailing in the retinal explants may be compared to those existing in nervous tissue during regeneration. Thus the general staining pattern for enzyme activities in the cultured nerve cells seems to be the same as that described in vivo for the retinal nerve cells. However the Golgi complex of the nerve cells in vitro never reached the complicated lamellar organization observed in vivo probably reflecting an incomplete development of the Golgi complex or a change in the cellular organization.

Granulated cells of mesenchymal origin varying from starshaped to spherical moved round among the other cells in the retinal cultures. Many of these cells showed high pinocytotic and phagocytotic activity (Hansson and Sourander 1964, Hansson 1965, 1966). A varying number fulfilled the criterions for mast cells (Olsson, Hansson and Sourander 1965). The histochemical demonstrated activity for several hydrolytic enzymes were localized to the cytoplasmic granules (i.e. acid phosphatases,  $\alpha$ -E leucyl naphthyl amide hydrolase, dinucleotidases and adenosine triphosphatase). The latter two reactions also outlined the cell membrane distinctly. All the granulated mesenchymal cells showed activity of the examined hydrolytic enzymes although the number of stained granules and the staining intensity varied between different cells. Previous histochemical studies in vivo have revealed similar properties for mast cells in subcutaneous tissue. The enzyme activity appeared in rat fetuses a few days before birth and increased rapidly during the next few weeks (Schauer 1964). Other studies both in vivo and in vitro have demonstrated high activity of these enzymes histochemically in macrophages (for review, see Jacoby 1965). The granulated mesenchymal cells in the retinal cultures showed an increase in the activity of the tested enzymes during the first two weeks. The staining intensity did not change markedly thereafter as far as could be demonstrated in the explants if they were not subjected to treatment with chemical agents (Hansson 1966).

In conclusion it may be stated that the AChE activity was possible to

demonstrate in the retinal cultures in the nerve cells of different types including their processes after about a week in vitro. At the same time there was a morphological development of the same cells. The results indicate that the nerve cells, which are immature at the time of explantation, develop and attain several of the characteristics of mature nerve cells. The same is true for the photoreceptor cells, as demonstrated by their chemical (Hansson 1966) and histochemical properties.

## SUMMARY

Tissue cultures of retinae from mainly young rats and rabbits were grown for histochemical demonstration of the presence of acetylcholine esterase and other hydrolytic enzymes. Several different methods for esterases were used in combination with inhibitors with known properties for studies on the specificity of the histochemical results obtained on the tissue cultures.

Acetylcholine esterase activity was demonstrated with the use of acetylthiocholine iodide as substrate and with either of the drugs  $4 \times 10^{-6}$  M Mipafox,  $1 \times 10^{-8}$  M IsoOMPA or  $3 \times 10^{-6}$  M Nu 683 as inhibitors of nonspecific cholinesterases both on the cell membrane and in the cytoplasm of the multipolar nerve cells and in some small nerve cells. The nerve cell processes showed high activities even at points of connections between different processes and between nerve cell processes and perikaryons. Other histochemical methods with  $\alpha$ -naphthyl acetate and naphthol AS-D acetate as substrates did not reveal any acetylcholine esterase activity. Indoxyl acetate was probably hydrolysed to a certain degree by acetylcholine esterase.

Acetylcholine esterase activity was not evident in the nerve cells until after several days in culture. During the same time there was a structural development of the nerve cells. The visual cells lacked acetylcholine esterase activity.

The proximal parts of the rod-like processes from the visual cells showed in culture a high activity for esterases, acid phosphatases, peptidase, leucyl-naphthyl amide hydrolase, dinucleotidases, pyrophosphatase and adenosine triphosphatase. These results are discussed and related to the conditions *in vivo*.

Granulated cells of mesenchymal origin also showed high activities of hydrolytic enzymes.

## ACKNOWLEDGMENTS

The author is indebted to Assoc Professor P Sourander and Assist Professor F Sjoqvist for their interest and valuable criticism, to Dr J C Keesey for reading the English manuscript, and to Miss Brita Nilsson and Miss Margareta Persson for skilful technical assistance

This study was supported by a grant from the University of Goteborg and a grant (K 66 870) to Assoc Prof P Sourander from the Swedish Medical Research Council

## REFERENCES

- ALLEN J M The properties of Golgi associated nucleoside diphosphatase and thiamine pyrophosphatase I Cytochemical analysis *J Histochem. Cytochem* 1963 *11* 529-541
- BARAK T and P J ANDERSON Histochemistry Theory Practice and Bibliography Hoeber Medical Division, New York 1963
- BARRON K D and T O TUNCBAY Phosphatase histochemistry of feline cervical spinal cord after brachial plexectomy *J Neuropath. exp Neurol* 1964 *23* 368-386
- BORNSTEIN M B Morphological development of neonatal mouse cerebral cortex in tissue culture p 1-11 In Neurological and electroencephalographic correlative studies in infancy edit. by P Kellaway and I Petersén Grunc and Stratton New York 1964
- BUNGE R H M B BUNGE and E R PETERSON An electron microscope study of cultured rat spinal cord *J Cell Biol* 1965 *24* 163-191
- CALLAS G and W HILD Electron microscopic observations of synaptic endings in cultures of mammalian central nervous tissue *Z Zellforsch* 1964 *63* 686-691
- COUTEAUX R The differentiation of synaptic areas *Proc Royal Soc. London* 1963 *158 B* 457-480
- CRAIN S M and M B BORNSTEIN Bioelectric activity of neonatal mouse cerebral cortex during growth and differentiation in tissue culture *Exper Neurol* 1964 *10* 425-450
- CRAIN S M and E R PETERSON Complex bioelectric activity in organized tissue cultures of spinal cord (human rat and chick) *J Cell comp Physiol* 1964 *64* 1-14
- DIEGENBACH P C Use of inhibitors in cholinesterase histochemistry *Nature* 1965 *207* 308
- EHINGER B Cholinesterases in ocular and orbital tissues of some mammals *Acta Univ Lundensis* 1966 *Secuo II* No 2
- EICHNER D Zur Histologie und Topochemie der Netzhaut des Menschen *Z Zellforsch* 1958 *48* 137-186
- ESILA R Histochemical and electrophoretic properties of cholinesterases and nonspecific esterases in the retina of some mammals including man *Acta Ophthal* 1963 *Suppl* 77
- FREDRICSSON B and F SJOQVIST A cytomorphological study of cholinesterase in sympathetic ganglia of the cat *Acta morph Neerl Scand* 1962 *5* 140-166
- GEIGER R S The behaviour of adult mammalian brain cells in culture *Intern Rev Neurobiol* 1963 *5* 1-52
- GEIGER R S and W G SYOVE Localization of cholinesterases in adult mammalian brain cell cultures *Int J Neuropharmacol* 1962 *1* 295-302
- GLACOBINI E Histochemical demonstration of AChE activity in isolated nerve cells *Acta physiol scand* 1956 *36* 276-290
- GOMORI G Microscopic histochemistry Univ Chicago Press Chicago 1952
- HANSSON H A A tissue culture study of inherited dystrophy of the retina in mice *Virchows Arch path. Anat.* 1965 *340* 69-83
- HANSSON H A Selective effects of metabolic inhibitors on retinal cultures *Exptl Eye Res* 1966 *5* 335-354
- HANSSON H A and P SOORANDER Studies on cultures of mammalian retina *Z Zellforsch* 1964 *62* 26-47
- HILD W Das Neuron In *Handbuch der mikroskopischen Anatomie des Menschen* Vol IV part 4 p 1-184 Ed by W v Mollendorff und W Bargmann Springer Verlag Berlin 1959
- HILD W Cell types and neuronal connections in cultures of mammalian central nervous tissue *Z Zellforsch* 1966 *69* 155-188
- HOLMSTEDT B A modification of the thiocholine method for the determination of cholinesterase II Histochemical application *Acta physiol scand* 1957 *40* 331-337

- HOLMSTEDT B and F SJOQVIST Some principles about histochemistry of cholinesterase with special reference to the thiocholine method *Bibl nat* 1961 2 1—10
- HARKONEN M Carboxylic esterases oxidative enzymes and catecholamines in the superior cervical ganglion of the rat and the effect of pre and postganglionic nerve division *Acta physiol scand* 1964 63 Suppl 237
- JACOBY F Macrophages In *Cells and tissues in culture* Vol II p 1 Ed by E N Willmer Academic Press London 1965
- KARCZMAR A G Ontogenesis of cholinesterases In *Handbuch der experimentellen Pharmakologie* Vol 15 p 129 Ed by A Heffter und W Heubner Springer Verlag Berlin 1963
- KOELLE G E Cytological distributions and physiological functions of cholinesterases In *Handbuch der experimentellen Pharmakologie* Vol 15 p 187 Ed by A Heffter und W Heubner Springer Verlag Berlin 1963
- MALMGREN H and B SYLVEN On the chemistry of the thiocholine method of Koelle *J Histochem Cytochem* 1955 3 441—445
- MURRAY M R Nervous tissue in vitro In *Cells and tissues in culture* Vol 2 p 373 Ed by E N Willmer Academic Press London 1965
- NOVIKOFF A B Lysosomes in the physiology and pathology of cells Contributions of staining methods In *Ciba Foundation Symposium Lysosomes* p 36 J & A Churchill Ltd London 1963
- NOVIKOFF A B and E GOLDFISCHER Nucleosidediphosphatase activity in the Golgi apparatus and its usefulness for cytological studies *Proc nat Acad Sci (Wash)* 1961 47 802—810
- OLSSON Y H A HANSSON and P SOURANDER Mast cells in cultures from nervous tissue *Acta neuropath* 1965 5 307—311
- PEARSE A G E *Histochemistry theoretical and applied* J & A Churchill Ltd London 1960
- PETERSON E R S M CRAIN and M R MURRAY Differentiation and prolonged maintenance of bioelectrically active spinal cord cultures (rat chick and human) *Z Zellforsch* 1965 66 130—154
- ROSEN J W Das Auge und seine Hilfsorgane In *Handbuch der mikroskopischen Anatomie des Menschen* Vol 3 part 4 p 26 Ed by W v Mollendorff und W Bargmann Springer Verlag Berlin 1964
- SCARFELLI D G and E L CRAIG The fine localization of nucleoside triphosphatase activity in the retina of the frog *J Cell Biol* 1963 17 279—288
- SCHAUER A *Die Mastzelle* G Fischer Verlag Stuttgart 1964
- SHANTHAVERAPPA T R and G H BOURNE Histochemical demonstration of thiamine pyrophosphatase and acid phosphatase in the Golgi region of the cells of the eye *J Anat* 1965 99 103—117
- SJOSTRAND F and S E NILSSON The structure of the rabbit retina as revealed by electron microscopy In *The rabbit in eye research* p 449 Ed by J H Prince Charles C Thomas Springfield 1964
- SJOSTRAND J Changes of nucleoside phosphatase activity in the hypoglossal nucleus during nerve regeneration *Acta physiol scand* 1966 67 219—228
- TORACK H M and R J BARNETT Nucleoside phosphatase activity in membranous fine structures of neurons and glia *J Histochem Cytochem* 1963 11 763—772
- WOLF M K Differentiation of neuronal types and synapses in myelinating cultures of mouse cerebellum *J Cell Biol* 1964 22 259—279











ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 70 SUPPLEMENTUM 289

AUTORADIOGRAPHIC STUDIES ON  
THE DISTRIBUTION OF C<sup>14</sup>-LABELLED  
ASCORBIC ACID  
AND DEHYDROASCORBIC ACID

BY

LARS HAMMARSTRÖM



ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 70 SUPPLEMENTUM 289

---

*From the Department of Pharmacology Royal Veterinary College Stockholm and the Research Institute  
of National Defence Dept 1 Sundbyberg Sweden*

AUTORADIOGRAPHIC STUDIES ON  
THE DISTRIBUTION OF C<sup>14</sup>-LABELLED  
ASCORBIC ACID  
AND DEHYDROASCORBIC ACID

BY

LARS HAMMARSTRÖM

STOCKHOLM 1966



## GENERAL INTRODUCTION

Ascorbic acid is widely distributed in the animal organism as shown by chemical determinations. The adrenal cortex, the pituitary and the corpora lutea are found to have the highest concentrations, whereas the concentration in blood and muscles has been found to be lower than in most other tissues (cf. Lloyd and Sinclair 1953).

For the demonstration of ascorbic acid in histological sections the silver mirror technique has most frequently been used (Bourne 1933, 1936; Giroud and Leblond 1934; Clara 1954). The method is based upon the reduction of silver nitrate without the action of light or heat. This property was considered to be specific for ascorbic acid. However, the specificity of the method has been strongly criticized. Besides ascorbic acid several other substances in the tissues have been claimed to reduce the silver nitrate (Clara 1954; Pearse 1961). The method has also been criticized with respect to localization since diffusion may occur before the precipitation of the silver grains (Danielli 1946; Pearse 1961).

The biochemical role for ascorbic acid in the organism is still obscure and a number of miscellaneous functions have been suggested. The studies have mainly been performed on experimentally deficient guinea pigs, which like man, monkeys and a few other species are unable to synthesize ascorbic acid (Chatterjee et al. 1961). In ascorbic acid deficiency it has been found that fibroblasts, odontoblasts and osteoblasts lose their capacity to form collagen. There are also abnormalities in the blood vessels leading to generalized haemorrhages. In addition to these classical signs of scurvy several other functions in the body seem to be impaired in a deficiency state. There are reports of disturbances in the metabolism of carbohydrates and of the aromatic amino acids phenylalanine and tyrosine and in the resorption and disposition of iron (cf. Follis 1958). Several of the symptoms have been observed in an advanced deficiency state when the basal metabolism of the organism is profoundly deranged and it is difficult to distinguish the functions that are directly dependent on the vitamin from those changes that are secondary to others or due to simple inanition.

Fully developed scurvy is rarely seen nowadays but slight ascorbic acid deficiency has been found to be rather common (Czok 1965). The early symptoms of ascorbic acid deficiency are not specific and the vitamin has been used for the therapy of several miscellaneous disorders (Ritzel 1965). Of special interest in dentistry is the possible relation between a subclinical scurvy and the periodontal diseases.

Several analogues of ascorbic acid have been tested for antiscorbutic





The physiological requirement of ascorbic acid has been extensively investigated. Yet it cannot be considered to be finally settled. The amount of ascorbic acid that has been found to prevent scurvy in humans is approximately 10–20 mg a day. The generally recommended daily allowance is 75 mg (Kraut 1965).

In guinea pigs the requirements seem to be considerably higher calculated on a weight basis. A daily dose of 6–8 mg/kg body weight seems to prevent scurvy and 30–80 mg/kg is an abundant safe daily dose for guinea pigs (Mannering 1949).

Administered ascorbic acid is well tolerated by the organism even when it is given in large amounts. In order to avoid the effects of the acidity of the vitamin large amounts are generally given in a neutralized solution or as a salt. Demole (1934) gave 5 g/kg body weight orally or 1 g/kg intravenously of ascorbic acid in a neutralized solution to mice and rats without any symptoms. Kieckebusch et al (1963) studied the chronic toxicity in rats of the vitamin given orally as sodium ascorbate. A daily dose corresponding to 6.5 g/kg body weight was well tolerated whereas a dose of 27.3 g/kg was toxic (77% mortality in 4 weeks). The low toxicity may in part be explained by the rapid excretion. When the concentration in blood plasma in humans exceeds 1.4–2 mg/100 ml the urinary excretion of ascorbic acid rapidly increases (Friedman et al 1940).

In the present investigation 9–18 mg/kg body weight of ascorbic acid was administered intravenously to adult male mice and to pregnant female mice. The distribution of the labelled ascorbic acid was studied at different intervals after injection ranging from 2 minutes to 6 days by means of whole body autoradiography (Ullberg 1954, 1958). In addition the distribution in some selected organs at 5 minutes and 4 hours after injection was studied in more detail by means of microautoradiography (Hammarstrom et al 1965). Ascorbic acid is easily soluble in water and in the methods used the tissues containing the labelled vitamin have not been in contact with any aqueous solution before the autoradiographic exposure.

## MATERIALS AND METHODS

### *Labelled compound*

Ascorbic acid 1 C<sup>14</sup> with a specific activity of 2.4 millicuries per millimole (mCi/mM) was obtained from New England Nuclear Corporation, Boston,

naturally occurring isomer has the highest activity Dehydro 1-ascorbic acid and some esters which metabolically can be converted to 1-ascorbic acid have about the same antiscorbutic activity as 1-ascorbic acid<sup>1</sup> The activity of all other substances tested is considerably lower (Brubacher and Vuilleumier, 1965) In the animal body ascorbic acid is mainly found in the reduced state (cf Lloyd and Sinclair, 1953)

Administration of ascorbic acid in amounts greatly in excess of the physiological requirements causes no demonstrable effects (Kieckebusch 1963 Grab 1965 Lang 1965) whereas injection of dehydroascorbic acid causes several specific symptoms Administration of dehydroascorbic acid has been shown to cause a temporarily increased blood pressure and increased motor activity, to raise higher nervous activity for several days and to induce a temporary or permanent diabetes (Patterson 1949 1950 Patterson and Mastin 1951, Wegmann 1958 Lyhs and Tegeler 1963) This may indicate a physiological role for dehydroascorbic acid it may be the active substance in some functions generally thought to be due to ascorbic acid

The distribution of injected dehydroascorbic acid is only fragmentarily known Some quantitative studies after injection of dehydroascorbic acid have however been performed It has been shown that dehydroascorbic acid penetrates more easily into the brain and eye than does ascorbic acid (Patterson and Mastin 1951 Martin 1961 Martin and Mecca 1961, Lyhs and Tegeler 1963) After entering these tissues it seems to be rapidly reduced to ascorbic acid Dehydroascorbic acid is more lipid soluble than ascorbic acid and it has been suggested to be the form for transport of the vitamin across cellular barriers (Patterson and Mastin 1951 Martin 1961 Martin and Mecca 1961) However this cannot be regarded as finally settled

The present autoradiographic investigation was undertaken in order to get more detailed information of the distribution of ascorbic acid and dehydroascorbic acid A better knowledge of these patterns might facilitate the understanding of the physiological functions of vitamin C

The tissue distribution in adult mice and suckling rats of C<sup>14</sup> labelled ascorbic acid and dehydroascorbic acid has been studied by whole body autoradiography according to the method described by Ullberg (1954 1958) In addition more detailed studies on the distribution in some selected tissues have been made with a recently developed microautoradiographic technique (Hammarström et al 1965) In order to be able to make more quantitative estimations of the autoradiograms impulse counting has been made on some organs Finally the chemical identity of the radioactive substances at different intervals after injection has been established by thin layer chromatography A preliminary report of this work has earlier been presented (Hammarström 1965)

# DISTRIBUTION OF ASCORBIC ACID 1-C<sup>14</sup> IN ADULT MICE

The physiological requirement of ascorbic acid has been extensively investigated. Yet it cannot be considered to be finally settled. The amount of ascorbic acid that has been found to prevent scurvy in humans is approximately 10—20 mg a day. The generally recommended daily allowance is 75 mg (Kraut, 1965).

In guinea pigs the requirements seem to be considerably higher calculated on a weight basis. A daily dose of 6—8 mg/kg body weight seems to prevent scurvy and 30—80 mg/kg is an abundant safe daily dose for guinea pigs (Mannering 1949).

Administered ascorbic acid is well tolerated by the organism even when it is given in large amounts. In order to avoid the effects of the acidity of the vitamin large amounts are generally given in a neutralized solution or as a salt. Demole (1934) gave 5 g/kg body weight orally or 1 g/kg intravenously of ascorbic acid in a neutralized solution to mice and rats without any symptoms. Kieckebusch et al (1963) studied the chronic toxicity in rats of the vitamin given orally as sodium ascorbate. A daily dose corresponding to 6.5 g/kg body weight was well tolerated whereas a dose of 27.3 g/kg was toxic (77 % mortality in 4 weeks). The low toxicity may in part be explained by the rapid excretion. When the concentration in blood plasma in humans exceeds 1.4—2 mg/100 ml the urinary excretion of ascorbic acid rapidly increases (Friedman et al 1940).

In the present investigation 9—18 mg/kg body weight of ascorbic acid was administered intravenously to adult male mice and to pregnant female mice. The distribution of the labelled ascorbic acid was studied at different intervals after injection ranging from 2 minutes to 6 days by means of whole body autoradiography (Ullberg 1954, 1958). In addition the distribution in some selected organs at 5 minutes and 4 hours after injection was studied in more detail by means of microautoradiography (Hammarström et al 1965). Ascorbic acid is easily soluble in water and in the methods used the tissues containing the labelled vitamin have not been in contact with any aqueous solution before the autoradiographic exposure.

## MATERIALS AND METHODS

### *Labelled compound*

Ascorbic acid-1 C<sup>14</sup> with a specific activity of 2.4 millicuries per millimole (mCi/mM) was obtained from New England Nuclear C.

Mass USA The radioactive compound was dissolved in redistilled water to give a concentration of 1.8 mg/ml (25  $\mu$ ci/ml)

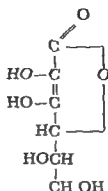


Fig. 1 Structural formula of ascorbic acid. The asterisk indicates the site of labelling.

### Animals

Adult white mice of the NMRI strain were used. The male mice weighed about 20 g and the female mice, which were in advanced (17–19 day) pregnancy, weighed about 40 g. For the whole body autoradiography, 5 male and 5 pregnant female mice were used, and in the microautoradiography experiments, 1 male and 1 pregnant female mouse were used.

### Diet

The mice were fed a complete pelleted mouse diet and water *ad lib*. The pellets were supplied by AB Ewos Södertälje, Sweden, and were composed of wheypowder 5%, expanded cereals 71%, soybean oil meal 9%, fish meal defatted 8%, soybean oil 2%, yeast 2%, lime stone 1%, vitamin concentrate 1%, trace elements 1%. Average analysis: dry matter, crude protein 20%, crude fat 4%, ash 5% (Ca 0.8%, P 0.6%), fiber 3%, N free extracts 68%. Water content 8%.

Vitamins added (per 100 grams): vitamin A 360 IU, vitamin D 36 IU, vitamin E 4.2 IU, thiamine 150 micrograms, riboflavin 300 micrograms, pyridoxine 500 micrograms, pantothenic acid 500 micrograms, niacin 1 milligram, choline chloride 0.1 gram.

Trace elements added (per cent): Fe 0.0016, Cu 0.00017, Co 0.000017, Mn 0.0016, I 0.00006, Zn 0.0126.

According to the supplier, the diet did not contain any vitamin C.

## *Whole body autoradiography*

Immediately after dissolving the ascorbic acid  $1\text{ C}^{14}$  in the redistilled water it was injected into a tail vein of the mice. Each animal was given 0.2 ml containing 0.36 mg ascorbic acid. After predetermined survival periods the animals were anaesthetized with ether and then immersed into a mixture of hexane and solid  $\text{CO}$  ( $-75^\circ\text{C}$ ). The female mice were sacrificed at 2 minutes, 20 minutes, 4 hours, 24 hours and 3 days after injection and the male mice were sacrificed at 5 minutes, 60 minutes, 24 hours, 4 days and 11 days after injection. After freezing they were mounted on a microscope stage in carboxy methyl cellulose mixed with water. In a freeze room ( $-10^\circ\text{C}$ ) sagittal sections through the whole animal were then cut at different levels. To obtain whole sections an adhesive tape (No. 810 Minnesota Mining and Manufacturing Co.) was applied to the section surface of the frozen specimen before cutting. The sections then adhered to the tape. Twenty and  $80\text{ }\mu$  thick sections were taken. The sections were freeze-dried at  $-10^\circ\text{C}$  after which they were allowed to reach room temperature in an air tight box. Autoradiograms were made by apposition of the sections against X-ray films (Structurix Gevaert Kodirex Kodak). After the exposure (2 weeks—2 months) the sections were separated from the film. The films were developed in Gevaert G 230 and fixed in Gevaert G 30 A. Finally the sections were stained with hematoxylin and eosin or toluidine blue and mounted on glass slides in Euparal (Flatters & Garnett Ltd Manchester England). The technique has been described in detail by Ullberg (1954, 1955).

## *Microautoradiography*

One male and 1 pregnant female mouse were injected intravenously with the same dose of ascorbic acid  $1\text{-C}^{14}$  as the mice used in the whole body autoradiography. Five minutes after injection specimens were taken from the male mouse and 4 hours after injection specimens were taken from the female mouse under ether anaesthesia. Specimens were taken from the following organs: eye, adrenal, spleen, ovary, pancreas, stomach, small intestine, sublingual and submaxillary salivary glands, parotid gland, thyroid and lung. Immediately after removal of the tissue specimens they were rapidly frozen in isopentane cooled with liquid nitrogen and then freeze-dried under vacuum ( $10^{-4}\text{ mm Hg}$ ) for 6 days. After freeze-drying the specimens were paraffin embedded under vacuum. Five micron thick sections of the paraffin embedded tissues were taken on a cellophane tape. A section of the tape was attached to the surface of the paraffin block. When sections were cut off adhering to the tape which thereby prevented the section from coming off and facilitated its further handling. Three to four sections were attached to the same piece of tape.

In the dark room nuclear emulsion plates (Ilford G 5) with an emulsion thickness of 10 microns were immersed into a solution of 10 per cent glycerin in absolute ethanol. The plates were then dried in a vertical position for 5—10 minutes allowing the alcohol to evaporate. The gelatin layer of the photographic emulsion was thus drenched with a small amount of glycerin which made it sticky enough to keep the sections attached to the emulsion surface. The pieces of tape carrying sections were then applied to the plates. The nuclear emulsion plates were left for exposure (1—6 weeks) under a slight pressure. After exposure the G 5 plates with the adhering pieces of tape were immersed into water for 5 minutes after which the cellophane backings of the tape were removed with a pair of forceps. Then the G 5 plates with the sections and the adhesive layer of the tape were passed through a series of increasing concentrations of ethanol (70% 96% and abs ethanol) and transferred to xylene. During 2 hours stay in xylene the adhesive of the tape was dissolved and the G 5 plates with adhering sections were passed through a series of decreasing concentrations of ethanol (abs ethanol 96% and 70%) before photographic development and fixing. After photographic processing and rinsing of the plates the sections were stained with hematoxylin and eosin, passed through a series of increasing concentrations of ethanol, transferred to xylene and mounted under coverslip with canada balsam and finally studied in the light microscope. The method has previously been described (Hammarstrom et al 1965).

## RESULTS

After an intravenous injection of  $C^{14}$  ascorbic acid the radioactivity rapidly left the blood and localized in the tissues. As early as 2 minutes after injection there was a considerable concentration in the adrenal cortex, choroid plexa, enamel of the incisors, intestinal mucosa, liver and kidney (Fig 51). A rapid uptake was also observed in the pituitary, in some cells in the thyroid, probably the parafollicular cells, and in the submaxillary salivary gland.

The distribution was markedly changed with time. The concentration in the liver, the kidney and intestinal mucosa decreased and the concentration in the brain slowly increased. After long survival periods the concentration in the brain was higher than in any other organ. There was also an uptake in the ganglia but it proceeded much more rapidly than in the brain. The concentration seemed to be highest in the sympathetic ganglia.

In lymphatic tissues and in some endocrine organs—the ovary, testis, pancreatic islets and adrenal medulla—there was a gradual increase. The concentration in those tissues was still low after injection.

Sup cerv  
Choroid plexa ganglion Thymus Heart blood Spleen Adre nal Kidney



Harder a gland Incisor Salivary gland Liver Intestines  
enamel

Fig 2 Autoradiogram of a mouse 5 minutes after an iv injection of ascorbic acid 1 C<sup>14</sup>. Note the uptake (light areas) in the adrenal cortex superior cervical ganglion and the enamel of the incisor. In the brain hardly any radioactivity is visible except in the choroid plexa.



Fig 3 Left Microautoradiogram (section + autoradiogram) of the salivary glands of a mouse 5 minutes after an iv injection of ascorbic acid 1 C<sup>14</sup>. Note the uptake (black grains) in the acini of the submaxillary gland. The salivary ducts seem to be almost free from radioactivity like the sublingual and parotid glands. Htx eosin (x 30).

Fig 4 Right Microautoradiogram (section + autoradiogram) of the submaxillary salivary gland of a mouse 4 hours after an iv injection of ascorbic acid 1 C<sup>14</sup>. Note accumulation (black grains) in the wall of salivary duct. Htx eosin (x 70).



Choroid  
plexa

Bone  
marrow

Bronchial  
walls

Pancreatic  
islets

Adrenal

Kidney

Ovary

Intestines

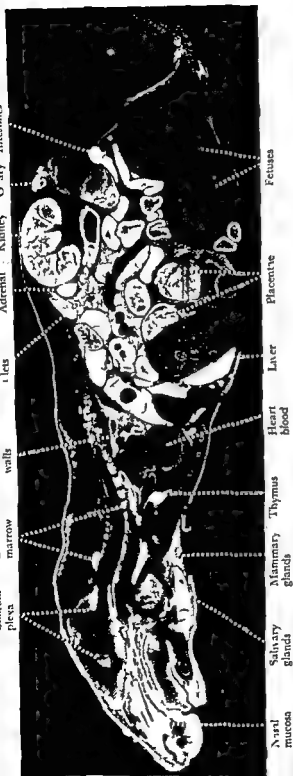


Fig. 3. Autoradiogram of a pregnant mouse 4 hours after an i.v. injection of ascorbic acid  $1\text{ Ci}^{14}$ . Note the accumulation (light areas) in both the adrenal cortex and medulla, the pancreatic islets and the ovary. Note also the penetration into the brain from the periphery and from the choroid plexa. The placentae have accumulated radioactivity but in the fetal tissues the concentration is low.

Nasal  
mucosa

Salivary  
glands

Mammary  
glands

Thymus

Heart  
blood

Liver

Placenta

Fetuses

Placentae

Spinal cord



Fig 6 Detail of Fig 5 Note the uptake in the fetal adrenals and retina Most fetal tissues have a low concentration The concentration in the fetal blood is not visible



Fig 7 Left Microautoradiogram (section + autoradiogram) of the oviduct of a pregnant mouse 4 hours after an iv injection of ascorbic acid  $1\text{ C}^{14}$  Note the uptake (black grains) in the muscular layer The mucosa seems to be devoid of radioactivity H&E eosin (x 80)

Fig 8 Right Detail of a whole body autoradiogram (upper) with the corresponding section (lower) of the ovary of a pregnant mouse 4 hours after an iv injection of ascorbic acid  $1\text{ C}^{14}$  Note the corpora lutea follicle walls and cumulus oophorus

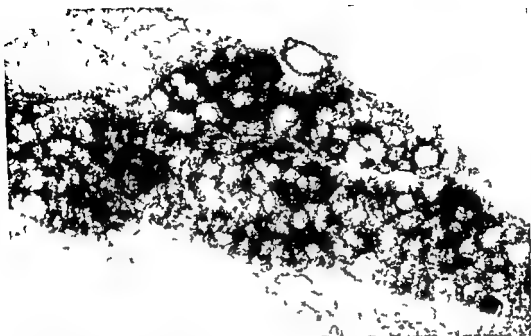


Fig 9 Microautoradiogram (section + autoradiogram) of the thyroid of a mouse 4 hours after an i.v. injection of ascorbic acid  $1\text{ C}^{14}$ . The highest concentration (black grains) is seen in some cells in and between the follicular walls probably the parafollicular cells. There is a low concentration in the ordinary follicular cells and hardly any radioactivity in the colloids. H+E eosin ( $\times 50$ )

The transfer of ascorbic acid to the fetus proceeded rather slowly. The fetal distribution pattern showed several similarities to that of the maternal tissues. Thus the adrenal initially showed the highest concentration and after long survival periods high concentration of radioactivity was observed also in the central nervous system of the fetus.

The distribution in the various tissues will be described in detail below.

#### *The circulatory system*

The concentration in the circulating blood rapidly decreased and was very low as early as 5 minutes after injection. No specific accumulation was observed in the walls of the blood vessels. In the myocardium radioactivity seemed to increase slowly but the concentration was rather low all the time. Also in the bone marrow there was a slow increase but the maximal concentration was rather low. In the lymphatic tissues—the white pulp of the spleen, the lymph nodules and the thymus—the concentration gradually increased and after long survival periods there was a rather high concentration compared to most other tissues except the brain and the spinal cord.

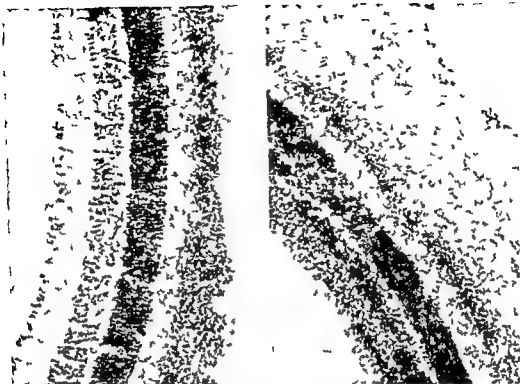
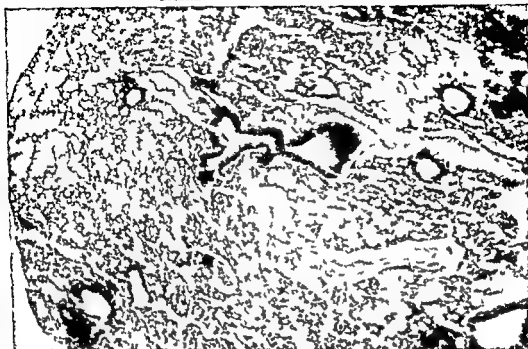


Fig 10 Left Microautoradiogram (section + autoradiogram) of a part of the eye of a mouse 5 minutes after an iv injection of ascorbic acid  $1\text{ C}^{14}$ . Note the accumulation (black grains) in the choroid. No radioactivity is visible in the retina. Htx eosin ( $\times 180$ )

Fig 11 Right Microautoradiogram (section + autoradiogram) of a part of the eye of a mouse 4 hours after an iv injection of ascorbic acid  $1\text{ C}^{14}$ . There is an accumulation (black grains) both in the choroid and in the retina. The ganglion cell layer and the inner nuclear layer seem to have the highest concentration in the retina. Htx eosin ( $\times 185$ )

### *The nervous system*

The radioactivity did not penetrate directly from the blood into the brain. In the *choroid plexa* however, a high concentration was observed as early as 2 minutes after injection. After 4 hours the radioactivity could be seen in the parts adjacent to the choroid plexa and in a peripheral zone close to the arachnoid space. The radioactivity continued to extend from these areas and 24 hours after injection radioactivity was observed in the whole brain. The concentration was higher in the gray matter than in the white matter. The highest final concentration in the brain seemed to be in the *hippocampus* and in the *cerebellar cortex*. In the *spinal cord* there was a similar slow uptake. Also in the *eye* there seemed to be some delay in the uptake but it proceeded more rapidly than in the brain. Four hours after injection the



Blood vessels

Fig. 12 Microautoradiogram (section + autoradiogram) of a lung of a mouse 4 hours after an iv injection of ascorbic acid  $1\text{ C}^{14}$ . A high concentration (black grains) can be noted in the bronchial epithelium. In the walls of the blood vessels no radioactivity is visible. Hix eosin ( $\times 40$ )

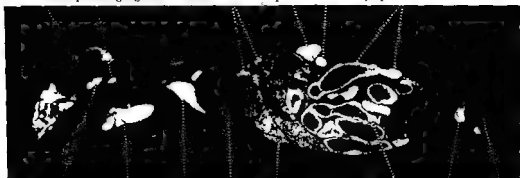
highest concentration in the eye was seen in the *choroid* and in the *retina* (Fig. 10 and 11). In the retina the highest concentration was seen in the ganglion cell layer followed by the inner and outer nuclear layers. Some radioactivity was found in the lens. In the *pineal body* radioactivity seemed to be concentrated directly after injection.

In the *peripheral nervous system* the radioactivity seemed to accumulate rapidly after injection. Radioactivity was observed in the *sympathetic ganglia*, *sensory ganglia* and also in the *myenteric plexa* in the gastro intestinal walls. The concentration was highest in the sympathetic ganglia. In the *peripheral nerves* the concentration was rather low.

### *The digestive system*

In the squamous epithelium of the *oral cavity*, the *oesophagus* and the oesophageal part of the *gastric mucosa* an uptake was observed. The secretory part of the gastric mucosa had a low concentration after short survival periods but it markedly increased with time. The mucosa of the *small intestine* rapidly concentrated large amounts of the radioactivity. In the mucosa of the *large*

Brain Sup cerv ganglion    Bronchial walls    Spleen Adrenal Lymph nodes



Pituitary Salivary glands Thymus Heart Liver Intestines Epididymus Testis  
blood

Fig 13 Autoradiogram of a male mouse 24 hours after an iv injection of ascorbic acid 1 C<sup>14</sup>. Note the uptake (light areas) in the brain pituitary adrenal cortex and medulla salivary glands and lymphatic tissues. The testis has a low concentration.

intestine the concentration was lower than in the small intestine. Microautoradiography did not reveal any specific localization in the secretory mucosa of the gastro intestinal tract. In the gastric mucosa the concentration was highest in the basal parts. The secretion into the lumen seemed to be insignificant.

The submaxillary salivary glands rapidly accumulated radioactivity which persisted for a long time and was still visible 6 days after injection. In the sublingual salivary glands the radioactivity did not appear until after some

Oesophageal  
part of gastric  
mucosa    Spleen Adrenal Ovary Fetus    Bone marrow  
Liver

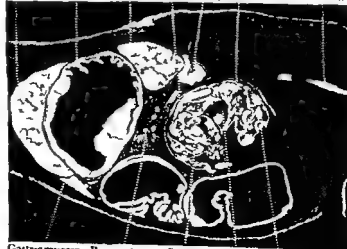


Fig 14 Detail of an autoradiogram of a pregnant mouse 24 hours after an iv injection of ascorbic acid 1 C<sup>14</sup>. Note the accumulation (light areas) in the ovary pancreatic islets and white pulp of the spleen. In the gastric mucosa the highest concentration is found in the basal parts. The adrenal the eye and the spinal cord seem to have the highest concentration of the fetal tissues in this section. In the fetal blood no radioactivity is visible.

Gastric mucosa    Pancreatic islets    Spinal cord    Heart blood    Eye

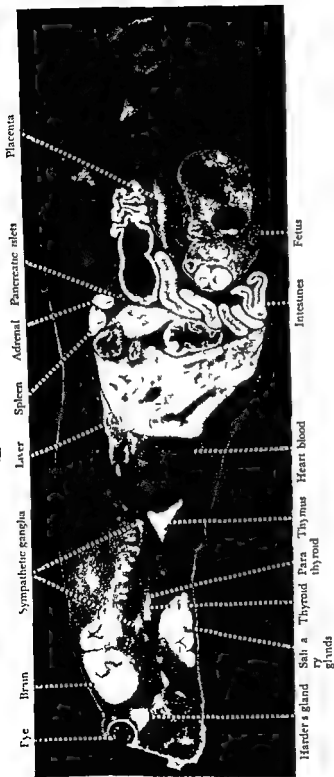


Fig 1b Autoradiogram of a pregnant mouse 3 days after an i.v. injection of ascorbic acid 1- $^{14}$ C. Note the accumulation (light areas) in both the maternal and fetal central nervous systems, eyes and adrenals. There is also an uptake in the maternal sympathetic ganglia, thyroid, parathyroid and pancreatic islets. The sublingual salivary gland has a higher concentration than the submaxillary salivary gland. No radioactivity is visible in the maternal and fetal blood.

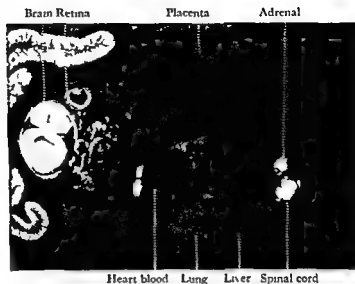


Fig. 16 Detail of Fig. 15 showing the distribution in the fetal tissues

latency. At 5 and 20 minutes the concentration was considerably lower than in the submaxillary gland but after 1 hour and 4 hours the mucous glands had a higher concentration than the submaxillary gland. After 3—4 days the differences had almost levelled out. In the *parotid gland* there was a slow increase of radioactivity to a moderate concentration. The microautoradiographic studies showed that 5 minutes after injection the radioactivity in the submaxillary salivary gland was localized to the acini. After 4 hours an accumulation was also seen in the walls of the salivary ducts and some radioactivity was found in the lumen of the ducts (Fig. 3 and 4). In the sublingual and parotid glands the distribution was more even at all the studied intervals. The *liver* showed an initially high concentration which then decreased with time more rapidly than most other tissues. The exocrine part of *pancreas* had a low concentration of radioactivity during the whole period of investigation. After 5 minutes some radioactivity was accumulated in the pancreatic ducts.

#### *The respiratory system*

The concentration in the *lungs* was initially about the same as that of the blood but the radioactivity in the lungs persisted much longer. In the *bronchial tracheal* and *nasal epithelium* radioactivity was rapidly accumulated. The concentration seemed to be maximal 4—24 hours after injection (Fig. 12).

#### *The endocrine system*

All the endocrine organs accumulated radioactivity. There were however great differences in their rate of uptake.



The *pituitary* rapidly accumulated radioactivity and a high concentration persisted for a long time. Four to 6 days after injection it was about the same as in the adjacent parts of the brain.

In the *thyroid* there was a moderate uptake in the follicle cells. In addition a marked accumulation was seen in some scattered cells, probably the para-follicular cells (Fig. 9). These cells which were unevenly distributed in the gland rapidly accumulated radioactivity and 6 days after injection an uneven distribution of radioactivity was still observed.

The *parathyroid* had a slightly higher concentration than the thyroid.

In the *pancreatic islets* a rather low concentration of radioactivity was seen shortly after injection. The radioactivity was unevenly distributed. After longer survival periods the concentration in the islets increased and the radioactivity was visible in most islet cells, possibly all of them. The radioactivity in the pancreatic islets was still visible 8 days after injection.

In the *adrenal cortex* a rapid accumulation was observed which seemed to be about the strongest in the body. Five minutes after injection the highest concentration was found in *zona glomerulosa* followed by *zona fasciculata* in a male mouse. Four hours after injection an uptake was also seen in *zona reticularis* of a pregnant mouse, whereas the radioactivity seemed to be low in *zona glomerulosa*. The concentration in the adrenal cortex decreased very slowly and was still rather strong 6 days after injection. The concentration in the *adrenal medulla* was moderate shortly after injection but it gradually increased. After 4 hours it seemed to be about the same as in the adrenal cortex and after long survival periods it was even slightly higher.

In the *ovaries* of a pregnant mouse the concentration was rather low and evenly distributed 2 minutes after injection. After 20 minutes the concentration had considerably increased. The strongest concentration was observed in *corpora lutea* and in the *walls of the follicles*. Also *cumulus oophorus* and the *ova* seemed to concentrate radioactivity (Fig. 8). The radioactivity in the ovary persisted for a long time and 3 days after injection it had about the same concentration as the white pulp of the spleen but less than that of the maternal and fetal central nervous systems.

The *testes* had a very slow uptake. The radioactivity was mainly localized in the interstitial parts.

### *The urinary system*

At short intervals after injection there was a strong concentration of radioactivity in the *kidney*, the *ureter* and in the *urinary bladder*. After the initial excretion phase some radioactivity persisted in the renal cortex. Twenty-four hours after injection however the concentration in the kidney was very low.

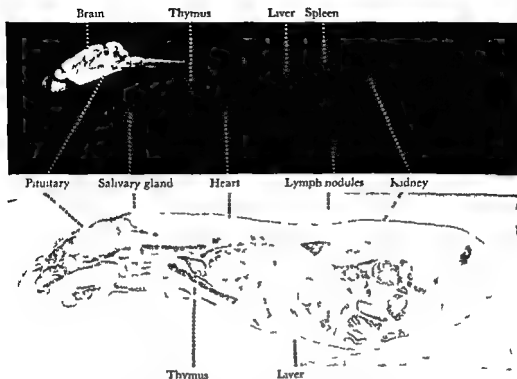


Fig 17 Autoradiogram (upper) with the corresponding section (lower) of a rat after an iv injection of ascorbic acid  $1\text{C}^{14}$ . Note the high concentration of radioactivity in the central nervous system. Also the lymphoid organs have a higher concentration of radioactivity than other tissues.

### The reproductive system

In the testes the radioactivity was mainly localized in the endocrine system. The uptake has been described in the endocrine system. The thymus followed that in the testis but the concentration in the seminal vesicles an accumulation was observed in the endocrine system. The distribution in the ovary has been described above. The moderate uptake of radioactivity in the muscular layer was observed in the mucosa (Fig 7).

### Muscles

The concentration in all the muscles was rather low. The smooth muscles and in the myocardium was higher than in the skeletal muscles.

### *Connective tissues and hard tissues*

The concentration in *fasciae* and *tendons* was rather low in the adult mice. In the cells of the *pericardial*, *pleural* and *peritoneal* cavities a high concentration was observed. The cells were of different types. Many of them were neutrophil leucocytes and some cells were mast cells as indicated by their metachromatic staining in toluidine blue.

In *compact bone* no radioactivity was visible except in the periosteum where a moderate concentration was observed. *Cartilage* had a low concentration which was evenly distributed. The *enamel* of the permanently growing incisors had a strong concentration throughout its thickness as early as 2 minutes after injection. In the *dentin* a zone of radioactivity could be seen adjacent to the dental pulp. The *pulp* of the incisors had a higher concentration than the circulating blood. In the *periodontal membranes* a moderate uptake was noted. The highest uptake seemed to be in the periosteum lining the dental alveoli.

### *Harder's gland*

This gland rapidly accumulated radioactivity to a high concentration which lasted for a long time.

### *The epidermis*

A moderate concentration was observed in the epidermis at all the intervals studied. Radioactivity was especially accumulated in the hair follicles.

### *The mammary glands*

In the mammary glands of the pregnant animals a moderate concentration of radioactivity was observed at all times studied.

### *The fetuses*

The transfer of labelled ascorbic acid to the fetuses was very slow. In all the pregnant mice studied the *placenta* had a moderate concentration. Four hours after injection some radioactivity had passed to the *fetuses* and it was evenly distributed in all the fetal tissues with some exceptions. The concentration in the *fetal blood* was very low and the *fetal adrenal* and *retina* had a higher uptake than the other fetal organs (Fig. 6). After 1 day and 3 days the concentration in the *fetal central nervous system* had increased (Fig. 14 and 16). Three days after injection the fetal brain, retina, spinal cord and adrenal seemed to have a higher concentration than the corresponding maternal organs. In the *fetal blood* no radioactivity was visible. There was no specific accumulation in the gastrointestinal tract, the salivary glands, the liver or in the kidney of the fetuses (Fig. 16).

## DISTRIBUTION OF DEHYDROASCORBIC ACID 1-C<sup>14</sup> IN ADULT MICE

It is now generally accepted that ascorbic acid normally occurs almost entirely in the reduced state in the body. Banerjee et al (1952) did not find any dehydroascorbic acid in the organs investigated and Damron et al (1952) found only trace amounts of the oxidized form. In muscles and blood however dehydroascorbic acid is reported to constitute 10—30 % of the total amount of vitamin C (ascorbic acid + dehydroascorbic acid) (Damron et al, 1952; Stewart et al 1953). The concentration of vitamin C in these tissues is however low and it is difficult to carry a solution of ascorbic acid through the analysis without producing small amounts of oxidized products.

In scurvy the ratio of dehydroascorbic acid to ascorbic acid has been reported to increase and the ratio has been called the «scurvitic index» (Martini et al 1935). However there are conflicting data whether there is an absolute or relative increase of dehydroascorbic acid (Martini et al, 1935; Banerjee et al 1952; Damron et al 1952).

Both *in vivo* and *in vitro* experiments have shown that dehydroascorbic acid is reduced to ascorbic acid by animal tissues (Johnson and Zilva 1934; Fox and Lewy 1936; Roe and Barnum 1936; Borsook et al, 1937; Schulze et al 1938; Penney and Zilva 1943; Todhunter et al 1950; Dayton et al 1959; 1966; Patterson and Mastin 1951; Martin 1961; Martin and Mecca 1961). Glutathione seems to be involved in the reduction (Borsook et al 1937). Recently an enzyme has been shown in erythrocytes and liver which catalyses this reaction (Hughes 1964).

Injection of dehydroascorbic acid causes several specific symptoms. Patterson and co workers have studied the effects of intravenously injected dehydroascorbic acid in rats. Following an injection they noticed a short period of hyperactivity followed by a collapse, an increased salivation and lacrimation which was sometimes followed by a porphyrin pigment, a temporarily increased blood pressure and a temporary or permanent diabetes. The hyperactivity could be controlled by anaesthesia. The salivation and lacrimation was inhibited by atropine. The blood pressure response was similar to that observed after injection of epinephrine and pretreatment with the adrenergic blocking drug «Dibenamine» produced a reversal of the response. Atropine sympathectomy with right splanchnicotomy and low cervical cordotomy all reduced the blood pressure response. Adrenalectomy did not affect the response. The hyperactivity and the increased blood pressure as well as the salivation and lacrimation were all considered to be due to a central stimulation of the autonomic system.

in the brain after an injection of dehydroascorbic acid and the following assumed change in acidity might cause a release of acetylcholine with a resultant stimulation of the brain (Patterson 1949 1950 Patterson and Mastin 1951) The diabetogenic effect was similar to that of alloxan As is the case for alloxan the animals could be protected if they were given sulfhydryl compounds such as glutathione cysteine or dimercaptopropanol shortly before the injection of dehydroascorbic acid The induced diabetes was suggested to be the result of an irreversible combination of dehydroascorbic acid with enzymatic sulfhydryl groups (Patterson 1949 Patterson and Lazarow 1950) Recently ultrastructural changes have been reported in the pancreatic islets of the rats following diabetogenic doses of dehydroascorbic acid (Merlini and Caramia 1965) There was a degranulation of the B cells and alterations in the organization of the granular endoplasmic reticulum and the mitochondria The A- and D cells seemed not to be affected Necrosis of the B cells as produced by alloxan was not seen The authors considered that the mechanism for the diabetogenic action of dehydroascorbic acid was not the same as that of alloxan and probably not due to an inhibition of sulfhydryl groups

The vasomotor effects of dehydroascorbic acid demonstrated in rats have later been shown to occur also in cats and guinea pigs (Wegmann 1958) In addition to the central stimulation observed by Patterson and Mastin (1951) he suggested that the assumed increased acidity also might release catecholamines from the peripheral nerves and the adrenals

Some effects of dehydroascorbic acid on the central nervous system have been investigated by Lyhs and Tegeler (1963) They found that an intracardial injection of dehydroascorbic acid to guinea pigs not only produced a general neuromotor excitement but also led to a rise of the higher nervous activity lasting for several days even with animals showing markedly weak conditioned reflexes

Most of the effects of dehydroascorbic acid decreased when the substance was injected repeatedly during a short period of time This has been found to be valid also for the toxicity In rats the LD<sub>50</sub> was about 320 mg/kg body weight intravenously Following a sublethal dose the rats tolerated a second dose which was 3 to 4 times as large as the initial dose After lethal doses of dehydroascorbic acid the animals died in respiratory failure similar to that observed after injection of lethal doses of epinephrine (Patterson and Mastin 1951) As little as 5 mg/kg of dehydroascorbic acid increased the blood pressure The hyperactivity was observed after a dose of 100—500 mg/kg and the diabetogenic dose was 1100 mg/kg Due to the toxicity this dose had to be preceded by a sublethal dose

In the present investigation 9—18 mg/kg was given intravenously to a series of male and pregnant female mice The animals were killed at different times after injection ranging from 2 minutes to 4 days and whole body

autoradiograms were made. In addition the distribution in some selected organs at 5 minutes and 4 hours after injection was studied by means of microautoradiography.

The labelled dehydroascorbic acid was obtained by oxidation of ascorbic acid-1- $C^{14}$  with benzoquinone as suggested by Moll and Vieters (1936) and according to the method described by Patterson (1950).

## MATERIALS AND METHODS

### *Labelled compounds*

7.3 mg ascorbic acid-1- $C^{14}$  with a specific activity of 2.4 mci/mM (New England Nuclear Corp.) was dissolved in 4 ml redistilled water. It was then shaken with an equal volume of ether containing benzoquinone (4.5 mg). The amount of benzoquinone was equivalent to the amount of ascorbic acid. After shaking for 15 minutes the ether was removed and the water solution was washed 5 times with an equal volume of ether. The water solution of dehydroascorbic acid 1- $C^{14}$  was then used immediately. Chromatographic control of the radiochemical purity of the solution according to the methods described later indicated that all the radioactivity represented dehydroascorbic acid 1- $C^{14}$ .

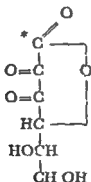


Fig. 18 Structural formula of dehydroascorbic acid. The asterisk indicates the site of labelling.

### *Animals*

Three male mice weighing about 20 g and 5 female mice in advanced (17–19 days) pregnancy weighing about 40 g were used. The mice were of the same strain as in the ascorbic acid 1- $C^{14}$  experiments and they were given

### *Whole body autoradiography*

Each mouse was given 5  $\mu\text{Ci}$  corresponding to about 0.37 mg (9–18 mg/kg body weight) dehydroascorbic acid 1- $\text{C}^{14}$  in 0.2 ml redistilled water intravenously in a tail vein. The male mice were then sacrificed 5 and 60 minutes and 4 days after injection and the female mice were sacrificed after 2 and 20 minutes, 4 and 24 hours and 3 days. The animals were sacrificed under ether anaesthesia by immersion into hexane mixed with solid  $\text{CO}_2$  ( $-75^\circ\text{C}$ ). The sectioning and autoradiographic procedures were identical with those used for ascorbic acid 1- $\text{C}^{14}$ . The exposure time was 2 weeks–2 months.

### *Microautoradiography*

Dehydroascorbic acid 1- $\text{C}^{14}$  of the same preparation as in the whole body experiments was used. One male mouse weighing 20 g and 1 female in advanced pregnancy were each injected in a tail vein with 5  $\mu\text{Ci}$  in 0.2 ml redistilled water corresponding to about 0.37 mg dehydroascorbic acid 1- $\text{C}^{14}$ . Five minutes after injection specimens were taken from the male mouse and 4 hours after injection specimens were taken from the female mouse. The specimens were taken under ether anaesthesia and samples from the following organs were taken: eye, adrenal, spleen, ovary, pancreas, stomach, small intestine, submaxillary and sublingual salivary glands, parotid gland, thyroid and lung. The specimens were rapidly frozen in isopentane chilled with liquid nitrogen. The freeze drying and subsequent treatment were identical to that described for microautoradiography with ascorbic acid 1- $\text{C}^{14}$ . Exposure time was 1–6 weeks.

## RESULTS

After an intravenous injection of  $\text{C}^{14}$  dehydroascorbic acid, observable amounts of the radioactivity remained in the circulating blood for several hours. The concentration in the brain and the spinal cord rapidly increased and as early as 2 minutes after injection it markedly exceeded that of the blood. It then remained high during the whole investigation period. A rapid and persistent uptake was also observed in the ganglia, the pituitary, the majority of the cells in the pancreatic islets and in the thyroid. In some other endocrine organs—the adrenal, the ovary and the testis—radioactivity was not accumulated until after some latency. Also in the lymphatic tissues there was a latency in the uptake.

After 3–4 days the highest concentrations were found in the central nervous system, pituitary, adrenal and in lymphatic tissues. At that time radioactivity was still observable also in the thyroid, pancreatic islets, ovary and testis.

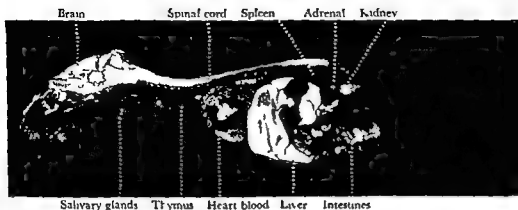


Fig 19 Autoradiogram of a mouse 5 minutes after an iv injection of dehydroascorbic acid 1  $C^{14}$ . Accumulation (light areas) is seen in the whole central nervous system. The concentration is low in the adrenal cortex. Radioactivity is still visible in the circulating blood.

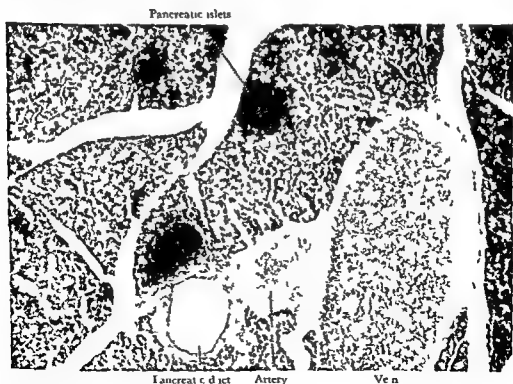


Fig 20 Microautoradiogram (stom + autoradiogram) of the pancreas of a mouse 5 minutes after an iv injection of dehydroascorbic acid 1  $C^{14}$ . Note the accumulation (black grains) in the pancreatic islets in comparison with the non visible concentration in the exocrine pancreas.



The transfer to the fetuses proceeded slowly and the distribution in the fetal tissues was similar to that observed after injection of  $C^{14}$ -ascorbic acid. The distribution in the various organs will be described in detail below.

#### *The circulatory system*

After an intravenous injection of  $C^{14}$ -dihydroascorbic acid some radioactivity remained in the blood for several hours and was still observable 4 hours after injection. No specific accumulation was observed in the walls of the blood vessels. In the myocardium the concentration initially was lower than that of the blood but it seemed to increase slowly and a low concentration was still observable 4 days after injection. In the bone marrow a moderate concentration of radioactivity persisted also when the concentration in the blood was no longer observable.

In the lymphatic tissues—the white pulp of the spleen, the lymph nodes and the thymus—the radioactivity gradually increased and persisted for a long time.

#### *The nervous system*

Immediately after injection radioactivity was accumulated in the nervous tissues—both in the central nervous system and in the ganglia. A high concentration then persisted during the entire investigation. The concentration was higher in the gray matter than in the white matter but the distribution was rather diffuse. After 3–4 days the concentration in the hippocampus and in the cerebellar cortex seemed to be higher than in other parts of the brain.

The radioactivity rapidly entered the eye and already 2 minutes after injection an uptake could be noted in the retina and also in the lens. At 5 minutes and 4 hours after injection the highest concentration in the retina seemed to be in the ganglion cell layer and in the inner nuclear layer.

The pineal body had about the same concentration as the gray matter. During the whole period of investigation radioactivity was accumulated in the choroid plexa. In the peripheral nervous system the sympathetic ganglia had a higher concentration than the spinal ganglia. Also in the myenteric plexa in the gastro-intestinal walls there was a rapid uptake (Fig. 21 and 22).

#### *The digestive system*

The stratified squamous epithelium of the oral and oesophageal mucosa and of the oesophageal part of the gastric mucosa accumulated radioactivity shortly after injection. The concentration in the secretory part of the gastric mucosa was initially low but it had increased after longer periods. The intestinal mucosa had a higher concentration than the gastric mucosa. Like the concentration in the gastric mucosa it increased and was rather high 4 hours after injection. The secretion into the lumen was insignificant.



Fig 21 Left Microautoradiogram (section + autoradiogram) of the duodenum of a mouse 5 minutes after an i.v. injection of dehydroascorbic acid  $1 \text{ C}^{14}$ . Note the accumulation (black grains) in the ganglia of Auerbach's plexus. Hix eosin ( $\times 7$ )

Fig 22 Right Detail of Fig 21 showing uptake in three ganglia of Auerbach's plexus ( $\times 164$ )

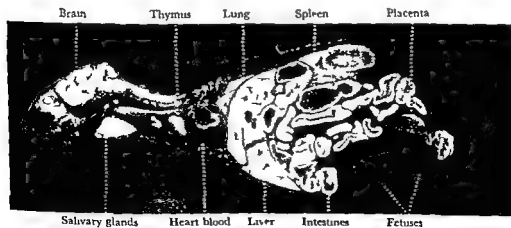


Fig 23 Autoradiogram of a pregnant mouse 4 hours after an i.v. injection of dehydroascorbic acid  $1 \text{ C}^{14}$ . Note the high concentration (light areas) in the brain and in the testes.

All the *salivary glands* had a low concentration shortly after injection of  $C^{14}$  dehydroascorbic acid. An accumulation was however observed in the walls of the salivary ducts and some radioactivity was also found in the lumen. The concentration in all the salivary glands gradually increased during the first few hours after injection. The submaxillary gland seemed to reach the highest concentration.

The *liver* had the highest concentration in the body shortly after injection. The radioactivity then gradually left the liver and after 4 days the concentration was rather low. In the exocrine part of the *pancreas* the concentration was rather low and evenly distributed after all the intervals studied.

### *The respiratory system*

The concentration in the *lungs* was initially about the same as that of the blood but the radioactivity in the lungs persisted for a longer time. Four days after injection some radioactivity still could be observed in the lungs. Also in the *nasal bronchial* and *tracheal epithelium* a persistent accumulation was observed.

### *The endocrine system*

The concentration in the *pituitary* was about the same as that observed in the adjacent parts of the brain during the whole investigation.

In the *thyroid* a moderate concentration of evenly distributed radioactivity was observed shortly after injection. In addition a strong accumulation in a few scattered cells, probably the parafollicular cells, was observed. This unevenly distributed radioactivity persisted for a long time after injection.

The concentration in the *parathyroid* was slightly higher than the average concentration in the thyroid.

The *pancreatic islets* rapidly accumulated radioactivity to a rather high concentration. The majority of the islet cells—possibly all of them—seemed to contain radioactivity (Fig. 20).

The *adrenal cortex* had not accumulated radioactivity 2 minutes after injection of  $C^{14}$  dehydroascorbic acid. Twenty minutes after injection the concentration slightly exceeded that of the blood and it then continued to increase for several hours. Four hours after injection the concentration seemed to be the highest in the body. The radioactivity was then mainly localized to *zona fasciculata* and *zona reticularis*. The *adrenal medulla* had a moderate concentration shortly after injection which exceeded that of the cortex. Like the cortex the concentration in the medulla increased. Four hours after injection it was slightly lower than in the cortex. Four days after injection the concentration in the cortex had decreased and the medulla had the highest concentration in the adrenal.

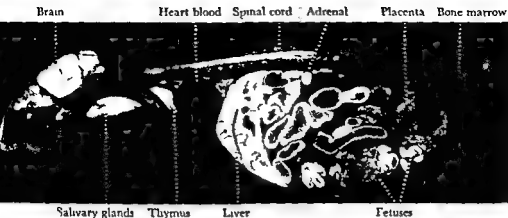


Fig. 94 Autoradiogram of a pregnant mouse 24 hours after an i.v. injection of dehydroascorbic acid  $C^{14}$ . Note the high concentration (light areas) in the maternal brain, adrenal cortex and medulla, salivary glands and lymphoid tissues. The concentration in the fetal brain is higher than in most other fetal tissues.

The *ovaries* of the pregnant mice accumulated radioactivity after some latency. Four hours after injection the concentration seemed to be about the same as after injection of  $C^{14}$  ascorbic acid. The localization was also similar to that of ascorbic acid.

The uptake in the *testes* was slow. The radioactivity was mainly localized in the interstitial parts.

#### *The urinary system*

The *kidney* did not accumulate as much radioactivity as after injection of  $C^{14}$  ascorbic acid. The moderate concentration in the kidney gradually decreased and after long survival periods the concentration in the kidney was low. At short intervals after injection radioactivity was found in the *ureter* and *urinary bladder*.

#### *The reproductive system and the muscles*

The uptake in the *reproductive system* and the *muscles* was very similar to that observed after injection of  $C^{14}$  ascorbic acid.

#### *Connective tissues and hard tissues*

The uptake in most connective tissues was rather low. In the *fasciae* and *tendons* a low concentration was observed. In similarity with the distribution of  $C^{14}$  ascorbic acid, some cells in the *pericardial*, *pleural* and *peritoneal cavities* also rapidly accumulated  $C^{14}$  dehydroascorbic acid.

In the *compact bone* of the adult mice no radioactivity was visible but in the *periosteum* an accumulation was observed. The *dentin* accumulated radio

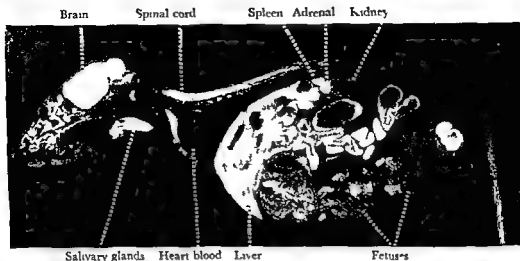


Fig 25 Autoradiogram of a pregnant mouse 3 days after an iv injection of dehydroascorbic acid 1  $C^{14}$ . The concentration (light areas) in the fetal adrenal and brain have now increased but still the concentration in the maternal brain and adrenal is higher

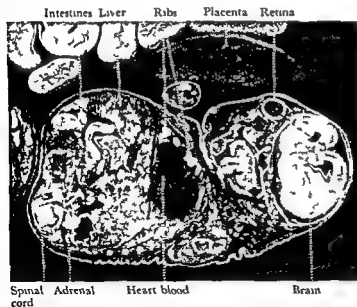


Fig 26 Detail of Fig 25 showing the distribution in the fetus. Compare the distribution with Fig 1

activity in a zone adjacent to the dental pulp. In the *enamel* of the growing incisor there was a latency in the uptake. One hour after injection a high concentration was observed throughout the thickness of the enamel. When the radioactivity had left the circulating blood a persistent moderate concentration was observed in the *dental pulp*. In the *periodontal membrane* the highest uptake seemed to be in the periosteum lining the alveoli.

### *Harder's gland*

*Harder's gland* had a high concentration of radioactivity at all intervals studied

### *The epidermis*

In the *epidermis* a moderate concentration was observed Especially in the hair follicles radioactivity was accumulated

### *The mammary glands*

In the *mammary glands* a moderate concentration was observed as early as 5 minutes after injection and radioactivity was still visible in these glands 3 days after injection

### *The fetuses*

After injection of  $C^{14}$ -dehydroascorbic acid the uptake in the placenta and fetal tissues was very similar to that observed after injection of  $C^{14}$ -ascorbic acid and the fetal tissues slowly accumulated radioactivity The concentration in the *placenta* initially was equal to that of the maternal blood but the radioactivity persisted longer in the placenta In the *fetuses* a low concentration of evenly distributed radioactivity was observed 4 hours after injection The concentration in the *fetal adrenal* and *retina* exceeded the average concentration and in the *fetal blood* no radioactivity was visible There was a slow increase in radioactivity in the *fetal central nervous tissues*



## DISTRIBUTION OF ASCORBIC ACID-1-C<sup>14</sup> AND DEHYDROASCORBIC ACID 1-C<sup>14</sup> IN SUCKLING RATS

Vitamin C plays an important role in growth processes and young individuals are known to require more ascorbic acid calculated on weight basis than the adult (Goldsmith 1961). During infancy there is a rapid synthesis of collagen in the growing skeleton and the symptoms of scurvy seem to be more dominated by skeletal changes in young individuals than in adults (Foliss 1959). In experimental scurvy young guinea pigs have frequently been used. A separate investigation on the distribution of ascorbic acid and dehydroascorbic acid in growing animals therefore seemed to be motivated.

C<sup>14</sup> labelled ascorbic acid or dehydroascorbic acid was given intraperitoneally to 10 days old rats and intravenously to 16 days old rats. Whole body autoradiograms were made and in addition a microautoradiographic investigation was made on the distribution in the head and a hind leg of the animals.

### MATERIALS AND METHODS

#### *Labelled compounds*

8.7 mg ascorbic acid 1-C<sup>14</sup> with a specific activity of 2.4 mCi/mM (New England Nuclear Corp.) was dissolved in 2 ml redistilled water immediately before use.

The same preparation of dehydroascorbic acid 1-C<sup>14</sup> was used as in the autoradiographic investigations with adult mice.

#### *Animals*

Eight 10 days old rats weighing about 20 g and six 16 days old rats of the Sprague Dawley strain weighing about 30 g were used in this investigation. After injection the sucklings were left with their mothers. They were given the same diet which has been described earlier.

#### *Whole body autoradiography*

Four of the 10 days old rats were each injected intraperitoneally with 5  $\mu$ Ci (0.37 mg) ascorbic acid 1-C<sup>14</sup> in 0.2 ml redistilled water and the remaining four of the 10 days old rats were each injected intraperitoneally with 5  $\mu$ Ci (0.37 mg) dehydroascorbic acid 1-C<sup>14</sup> in 0.2 ml redistilled water.

Fifteen minutes, 2 hours, 24 hours and 4 days after the injection one animal from each series was either anaesthetized and then sacrificed by *exsanguination* into a mixture of hexane and solid CO<sub>2</sub>. The sectioning and subdissection



body autoradiography was then performed according to the methods described earlier

The 16 days old rats were also divided into two series. Three rats were given 5  $\mu$ ci (0.37 mg) ascorbic acid  $1\text{ C}^{14}$  and the remaining 3 were given 5  $\mu$ ci (0.37 mg) dehydroascorbic acid  $1\text{ C}^{14}$  intravenously in a tail vein. Five minutes, 60 minutes and 24 hours after the injection one animal from each series was ether anaesthetized and then sacrificed by immersion into a mixture of hexane and solid  $\text{CO}_2$ . Twenty micron thick sections were taken on tape No. 810 (Minnesota Mining and Manufacturing Co.).

The subsequent autoradiography was then performed according to the methods described earlier.

### *Microautoradiography*

From all the animals in the experiment with growing rats 5  $\mu$  thick sections of the head and a hind leg of the animals were taken in addition to the 20  $\mu$  thick whole body sections. These sections were taken on tape No. 688 (Minnesota Mining and Manufacturing Co.). After drying in the freeze room the sections were applied to glycerin treated G 5 nuclear emulsion plates (Ilford emulsion thickness 10  $\mu$ ). The glycerin treatment of the G 5 plates was made according to the method described earlier.

After 2—4 weeks exposure the G 5 plates with adhering tape and sections were put in xylene 12—14 hours during which time the adhesive of the tape was dissolved and the pieces of tape fell off leaving the sections on the surface of the nuclear emulsion. The G 5 plates were then passed down through an alcohol series (absolute ethanol 96% and 70% ethanol) before they were developed, fixed and rinsed. After rinsing the sections still adhering to the plates were stained with hematoxylin and eosin, passed through an increasing alcohol series, transferred to xylene and mounted under cover slips in canada balsam.

## RESULTS

The distribution patterns after intravenous injection of  $\text{C}^{14}$  ascorbic acid to young rats showed great similarities with those seen in the adult mice. Thus there was a rapid accumulation of radioactivity in the adrenal cortex and a slow increase in the central nervous system. Also the distribution of intravenously injected dehydroascorbic acid in the young rats was similar to that seen in the adult mice. There was a rapid accumulation in the brain whereas the uptake in the adrenal cortex proceeded more slowly. However the distribution patterns in the young rats were different in some respects in comparison with those of the adult mice. The accumulation of radioactivity

in skeletal tissues was higher in the young rats and no specific uptake was observed in the pancreatic islets

After an intraperitoneal injection of  $C^{14}$  ascorbic acid the distribution seemed to be identical with that seen after an intravenous injection. After an intraperitoneal injection of  $C^{14}$  dehydroascorbic acid however the distribution pattern was more like that of intravenously injected  $C^{14}$  ascorbic acid. The rapid accumulation in the brain observed after intravenous injection of  $C^{14}$  dehydroascorbic acid did not occur. Instead there was a slow increase in the central nervous system. In addition to this "ascorbic acid pattern" there was a remarkably strong and long persisting concentration in the skeletal tissues (Fig. 45 and 46).

The distribution of radioactivity in the hard tissues will be described more in detail below.

### *Bone and cartilage*

Both after intravenous and intraperitoneal injection of  $C^{14}$  ascorbic acid and after intravenous injection of  $C^{14}$  dehydroascorbic acid there was a rapid accumulation of radioactivity in bone and cartilage. In the *cartilage* the radioactivity was evenly distributed. However in the epiphyseal plates a little higher concentration could be seen in the zone of cell multiplication and in the calcifying cartilage it was markedly higher. In the zone of lacunar enlargement the concentration was lower than the average.

The highest concentration in *bone* was seen in the primary bone trabeculae in the metaphysis and in the periosteum and endosteum in the diaphysis (Fig. 31). This distribution persisted for at least 4 days after injection. There was no indication of an incorporation of radioactivity as can be seen after injection of labelled bone minerals when the primary deposition lines can be seen deep in the bone after long survival periods. Also in the sutures of the bones of the skull a marked accumulation could be seen in the developing bone trabeculae (Fig. 34, 36).

### *The teeth*

In the dental tissues there was a rapid accumulation of radioactivity after intravenous injection of  $C^{14}$  ascorbic acid whereas there was a latency in the accumulation after intravenous injection of  $C^{14}$  dehydroascorbic acid (Fig. 40—43). The distribution patterns in the dental tissues however were very similar.

In the developing teeth a very diversified pattern could be seen. A description of the state of development of the teeth therefore seems warranted. In the 10 days old rats the enamel in some cusps of the first molars had reached its full thickness as indicated by the decrease in size of the ameloblasts (Fig. 34). In the other parts of the enamel of the first molars and in the

whole enamel areas of the second molars apposition of enamel matrix apparently still took place. In the 16 days old rats the enamel matrix of the first molars and also in large parts of the second molars seemed to have reached the final thickness.

$C^{14}$  ascorbic acid was found to be accumulated in a superficial zone of the enamel matrix in those areas where deposition of matrix was still taking place. In addition there was a pronounced uptake of radioactivity throughout the thickness of the enamel in those areas where matrix formation had apparently just ceased (Fig. 27, 28, 33 and 34). Four days after administration of  $C^{14}$  ascorbic acid to the 10 days old rats the radioactivity had left the initial accumulation areas and a sizable uptake throughout the thickness of the enamel was found more cervically in the first molars (Fig. 31 and 32). At that time a marked uptake could also be seen in the cusps of the second upper molar.

In the 16 days old rats no radioactivity appeared in the cusps of the first molars and in the cervical parts there was a low concentration throughout the thickness of the enamel. In the second molars a substantial accumulation could be seen throughout the thickness of the main part of the enamel. The third molars showed an uptake in the superficial parts of the enamel matrix (Fig. 42).

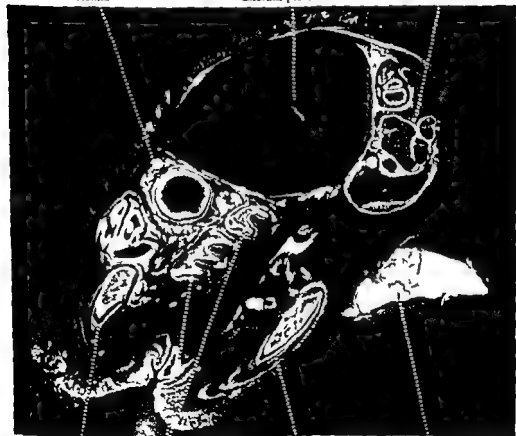
In the dentin a zone with a moderate concentration of radioactivity could be observed immediately adjacent to the predentin. The concentration in this zone decreased with time but it was localized adjacent to the predentin after all the intervals studied. In addition to this localization a zone of radioactivity could also be seen in the dentin close to the dentino enamel junction in those areas where the prominent uptake in the enamel was observed. As early as 5 minutes after an intravenous injection of  $C^{14}$  ascorbic acid this localization deep in the dentin was observed and the concentration was higher than in the pulpal zone of the dentin (Fig. 35). In some of the sections this accumulation could be seen in the tips of the cusps where the dentin is not covered by enamel. This shows that the autoradiographic blackening deep in the dentin was not due to radioactivity in the enamel (Fig. 33). Some radioactivity seemed to accumulate in the proximal part of the ameloblasts.

The dental pulp had a higher concentration of radioactivity than the circulating blood. The radioactivity was evenly distributed with the exception of the odontoblasts which had a higher concentration than the other cells of the pulp. The radioactivity seemed to be retained for a longer time in the pulps of the incisors than in those of the molars.

Brain      Scapula      Bronchial walls      Spleen      Kidney      Intestines



Dental pulp      Salivary glands      Heart blood      Liver      Gastric mucosa  
Retina      Choroid plexus      Inner ear



Upper incisor      Molars      Lower incisor      Salivary glands

Fig 27 Upper Aut radiogram of a 10 days old rat at 2 hours after an i.p. injection of ascorbic acid. Note the accumulation (light areas) in the gastric mucosa, bronchial walls and salivary glands. The concentration in the dental pulps is considerably higher than in the circulating blood.

Fig 28 Lower Aut radiogram of Fig 27. Note the marked accumulation in the enamel of the medial cusp of the first molar. There is also an accumulation in the superficial layer of the dentin and in the enamel. Note also the penetration into the brain from the choroid plexus.

Brain Sup cerv ganglion      Stellate ganglion      Adrenal      Pelvic bone



Salivary glands      Spinal ganglia      Thymus      Gastric mucosa      Urinary bladder  
Cerebellum      Sup cerv ganglion



Molars      Incisor      Salivary glands

Fig. 29 Upper. Autoradiogram of a young rat 24 hours after an i.p. injection of ascorbic acid (11). (The rat was 10 days old at the day of injection.) Note the difference in uptake (light areas) between the sympathetic and spinal ganglia. There is a high concentration in the adrenal, thymus, salivary glands and gastric mucosa.

Fig. 30 Lower. Detail of Fig. 29. Note the localization in the cerebellar cortex. The pulps of the incisors have a higher concentration than the pulps of the molars.

Brain

Lung

Spleen kidney

Pelvic bone



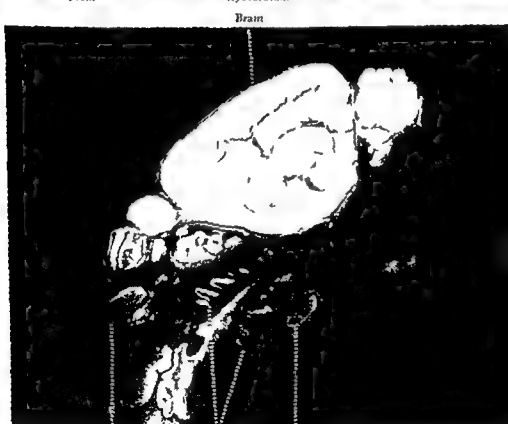
Teeth

Myocardium

Stomach

Intestines

Testis



Upper incisor

Molars

Lower incisor

Fig. 31 Upper Auto-gram of a young rat 4 days after an i.p. injection of ascorbic acid- $^{14}\text{C}$ . (The rat was 10 days old at the day of injection) The highest concentration (light areas) is seen in the brain. There is also a relatively high concentration in the testis.

Fig. 32 Lower Auto-gram of a young rat 4 days after an i.p. injection of ascorbic acid- $^{14}\text{C}$ . The accumulation in the enamel at the tip of the medial cusp of the first molar is far visible in Fig. 31 has now disappeared and the high concentration in the enamel is now located more cervically. In the second upper molar a high concentration is still visible in the cusps.





Fig 33 Left side upper Microautoradiogram (section + autoradiogram) of the upper first molar of a 10 days old rat at 2 hours after an i.p. injection of ascorbic acid  $1 \text{ C}^{14}$ . Note the high concentration (black grains) in the enamel at the tip of the medial cusp (E1) and the lower concentration in the superficial layer of the enamel matrix (E2). Note also the uptake in the dentin at the tip of the cusps (D1). An accumulation can also be seen in the dentin close to the pulp (D2). In the pulp the odontoblasts (O) have a higher concentration than the average of the pulp. Htx eosin ( $\times 40$ )

Fig 34 Left side lower Microautoradiogram (section + autoradiogram) of the medial cusp of the upper first molar of a 10 days old rat at 1, minutes after an i.p. injection of ascorbic acid  $1 \text{ C}^{14}$ . The exposure time has been short and the high uptake in the enamel is illustrated by the black grains at the tip of the cusp (arrow). Note the reduced height of the ameloblasts in this area indicating matrix completion. Htx eosin ( $\times 121$ )

Fig 35 Above Microautoradiogram (section + autoradiogram) of the second upper molar of a 16 days old rat at 5 minutes after an i.v. injection of ascorbic acid  $1 \text{ C}^{14}$ . Note the uptake (black grains) in the enamel and the adjacent parts of the dentin. The uptake in the dentin close to the predentin may be observed although the concentration there is lower than in the other localizations of the tooth. The arrows point to the two localizations in the dentin ( $\times 90$ )





Fig. 3b Microautoradiogram (section + autoradiogram) of a suture between two bones of the skull of a 10 days old rat 15 minutes after an i.p. injection of ascorbic acid 1  $C^{14}$ . Note the accumulation (black grains) at the ends of the trabeculae of the bone. No radioactivity is visible in the connective tissue between the two bones. Htx eosin ( $\times 35$ )



Fig. 3 Autoradiogram of a hind leg of a 10 days old rat 2 hours after an i.p. injection of ascorbic acid 1  $C^{14}$ . Note the accumulation (light areas) in the epiphyseal plate, in the trabeculae of the metatarsus and in the endosteum and periosteum in the diaphysis

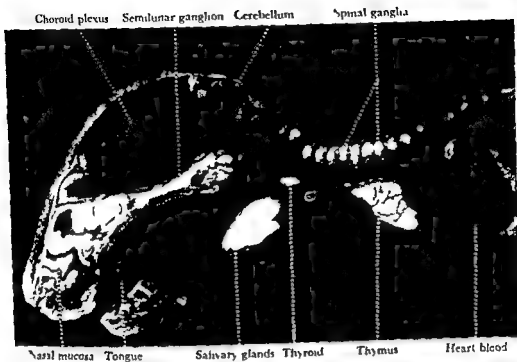


Fig. 39 Detail of an autoradiogram of a 10 days old rat 9 hours after an i.p. injection of ascorbic acid- $^{14}\text{C}$ . Note the accumulation (white areas) in the salivary glands, spinal ganglia and semilunar ganglion. Note also the pattern of penetration into the brain.

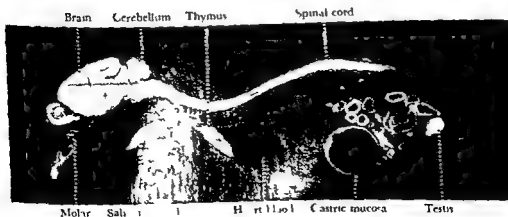


Fig. 38 Autoradiogram of a 14 days old rat 4 days after an i.p. injection of ascorbic acid- $^{14}\text{C}$ . (The rat was sacrificed 4 days after an i.p. injection) Note the accumulation (white areas) in the whole body, including the heart blood, testis and thymus.

Choroid plexus

Vertebrae



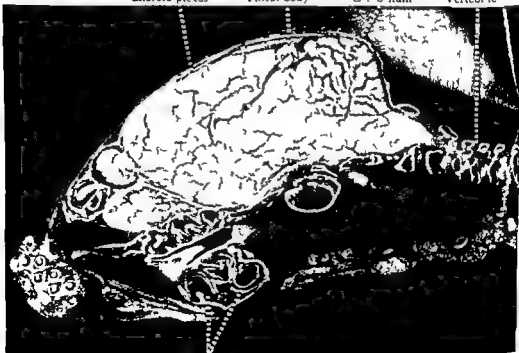
Teeth

Choroid plexus

Pineal body

C r b llum

Vertebrae



Teeth



**Fig 40** Left side upper Detail of an autoradiogram of a 16 days old rat 5 minutes after an iv injection of ascorbic acid  $1\text{ }C^{14}$  Note the accumulation (light areas) in the tooth enamel and in the choroid plexus

**Fig 41** Left side lower Detail of an autoradiogram of a 16 days old rat 5 minutes after an iv injection of dehydroascorbic acid  $1\text{ }C^{14}$  Note the accumulation (light areas) in the brain choroid plexus and pineal body In the teeth the concentration is low

**Fig 42** This is 1 upper Detail of Fig 40 (A) with the corresponding section (B) In the first molar there is a low uptake mainly in the cervical parts of the teeth In the second molars there is a considerable uptake in the whole enamel and also in the adjacent parts of the dentin

**Fig 43** This is 1 lower Detail of Fig 41 (A) with the corresponding section (B) There is a low concentration in the enamel of the teeth

Choroid plexus Pineal body



Upper incisor Pituitary Sublingual gland Submaxillary gland

Fig 44 Autoradiogram of a 16 days old rat at 5 minutes after an iv injection of ascorbic acid-1-C<sup>14</sup>. The picture illustrates the blood brain barrier to ascorbic acid. Accumulation (light areas) in the choroid plexus, pineal body and pituitary. Almost no radioactivity in the brain tissue. Note also the difference in up take in the salivary glands.

Brain Bone Adrenal



Salivary glands Heart blood Liver

Fig 45 Autoradiogram of a 10 days old rat 2 hours after an ip injection of dehydroascorbic acid-1-C<sup>14</sup>. Note the high concentration (light areas) in bone. This was not seen after the iv injection and probably indicating that some degradation products formed during the resorption have been accumulated there. Note also the low concentration in the brain compared with the one observed after intravenous administration.

Brain Cerebellum Thymus Adrenal



Incisors Salivary glands Bone Heart blood Liver

Fig 46 Autoradiogram of a 14 days old rat 4 days after an ip injection of dehydroascorbic acid-1-C<sup>14</sup>. (The rat was 10 days old at the day of injection). Note the persisting accumulation in bone and the increased concentration in the brain.

# CHROMATOGRAPHIC INVESTIGATION OF SELECTED TISSUES AFTER INJECTION OF ASCORBIC ACID 1-C<sup>14</sup> AND DEHYDROASCORBIC ACID 1-C<sup>14</sup>

Ascorbic acid is metabolized in the animal organism. By using ascorbic acid labelled in different positions it has been shown that a part of the molecule to some little extent may be incorporated in glycogen (Rudolff et al 1956; Chan et al 1958; Dayton et al 1959). After administration of ascorbic acid 1-C<sup>14</sup> however no significant amounts of radioactivity have been recovered in other components than ascorbic acid, dehydroascorbic acid and their degradation products (Burns et al 1951; Curtin and King 1955; Dayton et al 1959, 1966). The degradation of ascorbic acid and dehydroascorbic acid is considered to proceed to 2,3-diketo-1-gulonic acid, which then may either be decarboxylated and form carbon dioxide and pentonic acids or cleaved between carbons 2 and 3 yielding oxalic acid and possibly threonic acid. The catabolism of ascorbic acid has recently been reviewed by several authors (Burns 1960; Brubacher and Vuilleumier 1965; Degkwitz et al 1965).

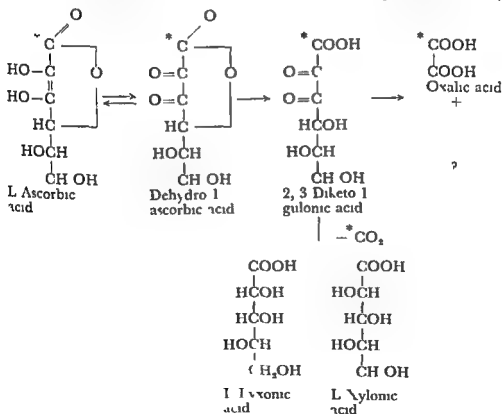


Fig 47 The most likely scheme for the catabolism of ascorbic acid. The asterisk indicates the site of labelling of the preparations used in the present investigation and the labelled possible metabolic

The conversion of ascorbic acid to urinary oxalate has been shown in man guinea pig and rat. The mechanism by which oxalate is formed is unknown. Attempts to demonstrate significant conversion of ascorbic acid to oxalate in rat and guinea pig tissues have not been successful (Burns 1960). The amount of ascorbic acid converted to urinary oxalate is rather small. Less than 2 per cent per day of the administered ascorbic acid  $1\text{-C}^{14}$  has been recovered as urinary  $\text{C}^{14}$ -oxalate (Burns et al. 1951, 1956; Lamden and Chrvstowski 1954; Curtin and King 1955; Dayton et al. 1959).

In both rats and guinea pigs there is a considerable conversion of ascorbic acid to carbon dioxide (Burns et al. 1951, 1954; Curtin and King 1955; Dayton et al. 1959, 1966; Salomon 1962). There are conflicting data however concerning the existence of this degradation pathway in man (Hellman and Burns 1958; Baker et al. 1962; Abt et al. 1963; von Schuching and Abt 1965).

Rather few qualitative tissue analysis have been made after administration of labelled ascorbic acid or dehydroascorbic acid and those studies have mainly been made 24 hours after intraperitoneal or oral administration. A special study was therefore desirable to establish what the radioactivity might represent in some of the organs which showed a marked accumulation in the autoradiographic studies. Thin layer chromatography was found to be a suitable method.

Both ascorbic acid and dehydroascorbic acid easily decompose during the analytical procedure. The decomposition is accelerated at a neutral or alkaline pH and in presence of ions of heavy metals especially those of copper and iron. In absence of these metals both ascorbic acid and dehydroascorbic acid are rather stable at pH 2–3 (Prochazka 1958; Heimann and Heimann 1965). Metaphosphoric acid has been shown to have a stabilizing effect upon ascorbic acid in solution (Musulin and King 1936; Bessey 1938; Fujisawa et al. 1963). In the present investigation metaphosphoric acid has been used both in the extraction solution and in the chromatographic plates. The application of the radioactive samples onto the chromatographic plates as well as the chromatography have been performed under nitrogen.

## MATERIALS AND METHODS

### *Labelled compounds*

Ascorbic acid  $1\text{-C}^{14}$  with a specific activity of 6.64 mCi/mM was obtained from New England Nuclear Corp. 1.2 mg of the labelled ascorbic acid was dissolved in 2.0 ml redistilled water. Half of it (1.0 ml) was oxidized to dehydroascorbic acid with an equivalent amount of benzoquinone (0.37 mg) in 1.0 ml ether according to the method earlier described. The purity of the

radioactive substances was controlled by thin layer chromatography and subsequent autoradiography as described below. The solution of ascorbic acid  $1\text{-C}^{14}$  showed only one radioactivity spot the  $R_f$  value of which corresponded to ascorbic acid whereas the solution of dehydroascorbic acid  $1\text{-C}^{14}$  showed two spots. One of them had an  $R_f$  value corresponding to dehydroascorbic acid containing 86 per cent of the applied radioactivity and the other spot had an  $R_f$  value corresponding to 2,3-diketogulonic acid containing 5 per cent of the applied radioactivity. Nine per cent remained at the origin (Fig 48).

### *Animal experiments*

Eight male mice weighing about 20 g were used. The mice were of the same strain as those used in the autoradiographic investigations and they were given the same diet. Four mice were given 0.15 mg ascorbic acid  $1\text{-C}^{14}$  and the remaining 4 mice were given 0.15 mg dehydroascorbic acid- $1\text{-C}^{14}$ . The substances which were dissolved in redistilled water were given intravenously in a tail vein and the dose corresponded to 7.5 mg/kg body weight (6  $\mu\text{Ci}$  per animal). At 5 minutes, 4 hours, 24 hours and 4 days after the injection one mouse of each series was killed by stretching the spine. The adrenals, liver, spleen, kidney, salivary glands (submaxillary and sublingual salivary glands) and brain were removed and homogenized in 15 ml Potter Elvehjem homogenizers.

The organs were homogenized in an extraction solution which was prepared as follows: 1.05 g citric acid was dissolved in 10 ml 1.0 N sodium hydroxide and diluted to a volume of 125 ml with redistilled water. To this was added 25 ml 3% metaphosphoric acid and 1.54 g trichloroacetic acid (Musulin and King 1936; Bessey 1938).

The liver, spleen, kidney, salivary glands and brain were each homogenized in 10 ml and the adrenals in 0.5 ml of the extraction solution. After homogenization they were centrifuged at  $1100 \times g$  for 10 minutes. Radioactivity assay with a GM counter of the sediment showed that practically all of the radioactivity of the tissues had been extracted.

The supernatant fluid was shaken with 0.5 ml benzene in order to avoid tailing of the chromatogram due to the extracted lipids. No radioactivity appeared in this benzene phase.

### *Chromatography*

About 30  $\mu\text{l}$  of each extract was spotted under nitrogen on 250  $\mu$  thick silica gel thin layer plates for chromatography. The plates were prepared as described by Stahl (1962). Silica gel H and 3% metaphosphoric acid was used for the preparation. The plates were activated by heating at  $120^\circ\text{C}$  for 1 hour.



The solvent system for thin layer chromatography found to give the best separation of possible labelled metabolites was benzene-methanol-acetic acid (70:20:5). The development of the chromatogram was performed under nitrogen. The front was allowed to run approximately 16 cm. Development time for the chromatograms was about 50 minutes. Non-radioactive ascorbic acid and dehydroascorbic acid were always run on each plate as reference substances. After the development of the chromatograms they were located by spraying the plates with 0.1% 2,6-dichlorophenol indophenol in 96% ethanol (w/v) and 0.1% 2,4-dinitrophenylhydrazine in 96% ethanol (w/v) to which had been added 1 ml concentrated HCl.

The radioactive compounds were located by autoradiography. The plates were pressed against Kodirex X-ray film (Kodak) with an intervening mylar film. Exposure time was 2 months. Then radioactive spots were scraped with a razor blade into liquid scintillation vials and counted in a Packard Tri-Carb liquid scintillation spectrometer as a suspension in 15 ml of 0.1% 2,5-diphenylloxazole + 0.03% 1,4-bis(2-(4-methyl-5-phenylloxazolyl)) benzene + 4% silica gel (Aerosil) in toluene (Snyder and Stephens, 1962). Correction for quenching was made by using the external radiation source of the spectrometer.

The approximate  $R_f$  value of ascorbic acid was found to be 0.80 which is in accordance with Stahl (1962) and the approximate  $R_f$  values of possible labelled metabolites of ascorbic acid  $1-C^{14}$  were found to be: hydroascorbic acid 0.45, 2,3-diketogulonic acid 0.20 and oxalic acid 0. It was not possible to obtain reproducible  $R_f$  values from plate to plate. A stream of nitrogen through the chromatograph chamber seemed to decrease the  $R_f$  values.

For the obtaining of the  $R_f$  values of the possible metabolites the following substances were used: dehydroascorbic acid prepared both with benzoquinone according to Patterson (1950) and with norit according to Roe and Kuetting (1949); 2,3-diketogulonic acid prepared according to Penney and Silva (1954) and finally  $C^{14}$  oxalic acid (The Radiochemical Centre, Amersham, England). Ascorbic acid  $1-C^{14}$  and dehydroascorbic acid  $1-C^{14}$  were also used. In the experiments 5–10% of the spotted radioactivity remained at the origin.

## RESULTS

Only two spots except the one at the origin were obtained in all the chromatograms of extracts from the adrenal, liver, spleen, kidney, salivary gland and brain (Fig. 48 and 49). These spots had the same  $R_f$  values as ascorbic acid and dehydroascorbic acid. The main part of the radioactivity had the same  $R_f$  value as ascorbic acid regardless of which one of the two vitamins

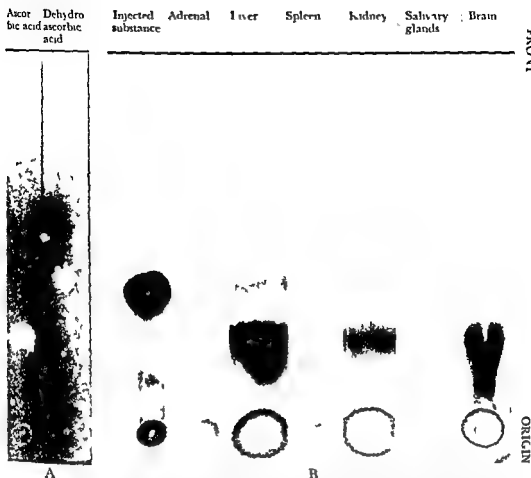


Fig. 48 Radiochromatogram of tissue extracts of a mouse 5 minutes after an iv injection of dehydroascorbic acid  $1\text{ C}^{14}$ . Photograph (A) of the non radioactive reference substances after spraying with 2.6 dichlorophenolindophenol and 2.4 dinitrophenylhydrazine. Autoradiogram (B) of the same plate. Almost all of the injected dehydroascorbic acid  $1\text{ C}^{14}$  has been reduced to ascorbic acid. Solvent system: benzene-methanol-aceton-acetic acid (70:20:5:5).

forms that had been injected. The ratio between the two spots seemed to be the same in all the tissues investigated and at all the various intervals after injection.

The amount of radioactivity in most of the spots with the same  $R_f$  value as dehydroascorbic acid generally were too low to be counted with any accuracy. An exception was the spots obtained from liver and kidney of the mice killed 5 minutes and 4 hours after injection of either one of the two vitamin forms. In these samples the spots with the same  $R_f$  value as dehydroascorbic acid contained approximately 90 per cent of the spotted radioactivity. Approximately 90 per cent had the same  $R_f$  value as ascorbic acid.

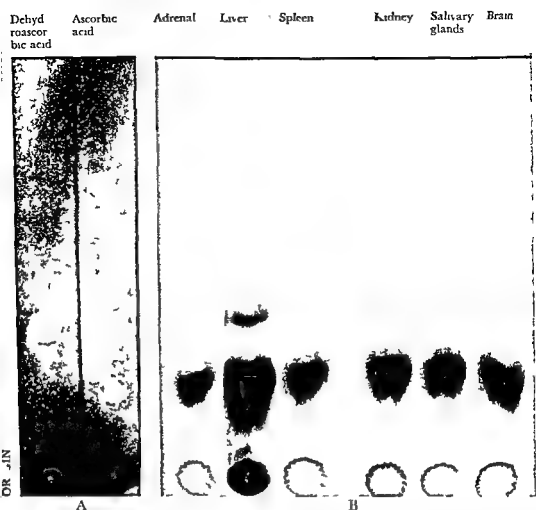


Fig. 49 Radiochromatogram of tissue extracts of a mouse 4 hours after an iv injection of ascorbic acid- $C^{14}$ . Photograph (A) of the reference substances. Autoradiogram (B) of the same plate. It is approximately the same ratio between the amounts of dehydroascorbic acid- $C^{14}$  and ascorbic acid- $C^{14}$  as was seen in Fig. 48. Solvent system: benzene-methanol-acetic acid (70:30:5:5).

## COMPARISON OF THE DISTRIBUTION PATTERNS OF ASCORBIC ACID 1 C<sup>14</sup> AND DEHYDROASCORBIC ACID 1 C<sup>14</sup> AS DETERMINED BY IMPULSE COUNTING

In order to be able to make a more quantitative evaluation of the autoradiographic pictures and to facilitate the comparison of the distribution patterns of the reduced and oxidized forms of vitamin C it was desirable to measure the concentration of radioactivity in some organs of mice by impulse counting at different times after an intravenous injection of the labelled substances.

The distribution of C<sup>14</sup>-labelled ascorbic acid in the animal body has earlier been studied by means of impulse counting. Burns et al (1951) studied the distribution in guinea pigs 24 hours after an oral or intraperitoneal injection of ascorbic acid 1 C<sup>14</sup>. They found the highest concentration in the adrenal and the lowest in the brain, kidney and heart. No marked differences were found in the distribution of the radioactivity in the organs and tissues of normal and scorbutic guinea pigs.

In the present study the adrenals, brain, kidney, liver, salivary glands and spleen were selected for impulse counting. The mice were injected intravenously and different intervals from 5 minutes to 4 days after injection the animals were killed and the organs immediately removed and homogenized. For the extraction the same solution was used as in the qualitative studies. Aliquots of the extracts were counted in a Packard Tri Carb liquid scintillation spectrometer.

### MATERIAL AND METHODS

#### *Labelled compounds*

37 mg ascorbic acid 1 C<sup>14</sup> with a specific activity of 24 mCi/mM was dissolved in 9.2 ml redistilled water. 5.5 ml of this solution was taken for the preparation of dehydroascorbic acid 1 C<sup>14</sup> with benzoquinone according to methods earlier described. Aliquots of the solutions of ascorbic acid 1 C<sup>14</sup> and dehydroascorbic acid 1 C<sup>14</sup> were taken for impulse counting. Chromatographic control of the purity according to the method earlier described indicated that all the radioactivity of the two solutions represented ascorbic acid 1 C<sup>14</sup> and dehydroascorbic acid 1 C<sup>14</sup> respectively.

#### *Animal experiments*

Sixteen male mice weighing 20–25 g were injected with 0.2 ml (0.08 mg 1 µCi) ascorbic acid 1 C<sup>14</sup> and 24 male of the same weight were injected

ted with 0.2 ml (0.08 mg 1  $\mu$ ci) dehydroascorbic acid-1  $C^{14}$ . The substances were injected in a tail vein. At 5 minutes, 30 minutes, 60 minutes, 2 hours, 4 hours, 24 hours, 2 days and 4 days after injection 2 of the animals injected with ascorbic acid 1- $C^{14}$  and 3 of the animals injected with dehydroascorbic acid 1  $C^{14}$  were killed by stretching the spine. The adrenals, liver, spleen, kidney, salivary glands (sublingual and submaxillary glands) and brain were removed, weighed and homogenized in 3 ml of an extraction solution of the same composition as that used in the chromatographic investigations. The corresponding organs from 2 and 3 animals respectively which were killed at the same interval after injection were pooled.

#### *Extraction and radioactivity measurements*

The organs were homogenized in Potter-Elvehjem homogenizers and then centrifuged at  $1100 \times g$  for 10 minutes. Radioactivity assay was then performed by liquid scintillation of 0.1 ml aliquots of the supernatants. Each 0.1 ml aliquot was added to a tricarb vial containing 3 ml ethanol and 7 ml toluene-PPO (5 g diphenyloxazole in 1 l toluene). The external standard of the spectrometer showed that no correction for quenching was needed. The values obtained by the liquid scintillation assays were calculated to give the concentration of radioactivity per gram of the tissue as percentages of the injected dose per gram body weight.

The sediments obtained at the centrifugation of the organs were assayed in a GM counter.

## RESULTS AND COMMENTS

The radioactivity assays on the washed sediments showed that practically all of the radioactivity was extracted. The results obtained by the liquid scintillation assays on the extracted radioactivity are condensed in the diagrams in Fig. 50.

The results of the impulse counting seem to be in accordance with the autoradiographic results and the differences between the distribution patterns observed in the autoradiographic investigation have been further supported.

The uptake in the brain at 5 minutes after injection of  $C^{14}$  dehydroascorbic acid was 10 times that noted after injection of  $C^{14}$  ascorbic acid. This ratio slowly decreased and as long as 4 days after injection there was still a difference. The « $C^{14}$  dehydroascorbic acid curve» also showed a remarkable peak at 24 hours after injection. This was also noted in a similar preliminary investigation. The simultaneous maximum in the « $C^{14}$  ascorbic acid curve» indicates that it may be due to an uptake of labelled ascorbic acid emanating from the injected dehydroascorbic acid which has been reduced somewhere else in the body.

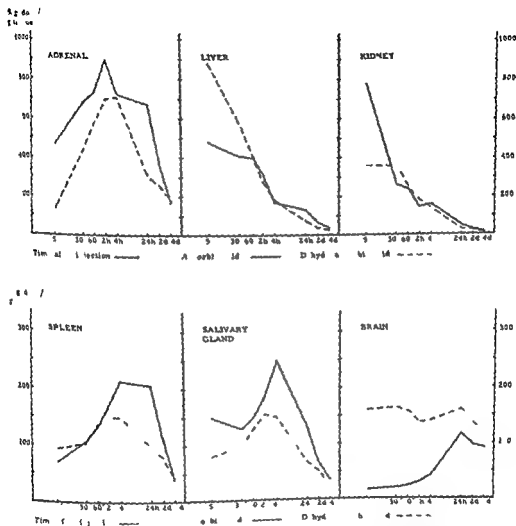


Fig 10 Radioactivity in adrenal liver kidney spleen salivary glands (sublingual and submaxillary glands) and brain of mice at different intervals after iv injection of ascorbic acid  $C^{14}$  or dehydroascorbic acid  $C^{14}$ . The ordinates give the contents per gram tissue as percentages of the dose given per gram animal. The same dose of ascorbic acid  $C^{14}$  as of dehydroascorbic acid  $C^{14}$  was given.

The concentration in the adrenal was found to be more than 3 times higher at 5 minutes after injection of  $C^{14}$  ascorbic acid than after  $C^{14}$  dehydroascorbic acid. The autoradiographic investigation indicated a still greater difference at 2 minutes after injection. The uptake in the adrenal medulla seems to be the same regardless of which one of the two compounds that had been injected and the difference in concentration in the whole adrenal apparently reflects the different concentrations in the adrenal cortex.

Brain

Choroid plexus

Liver Spleen Adrenal Kidney



Incisor Pituitary

Heart blood

Placentae Fetuses

Brain

Liver Spleen Adrenal Kidney



Salivary gland

Heart blood

Placenta

Fetuses

Fig 51 Upper Autoradiogram of a pregnant mouse 2 minutes after an iv injection of ascorbic acid-14C

Fig 52 Lower Autoradiogram of a pregnant mouse 5 minutes after an iv injection of dehydroascorbic acid-14C

Note the differences: a high concentration (light areas) in the adrenal cortex and no visible uptake in the brain except in the choroid plexa after injection of ascorbic acid-14C; a very low concentration in the adrenal cortex and a high concentration in the brain after injection of dehydroascorbic acid-14C. The concentration is low in the spleen of both animals and no transfer across the placenta seems to have occurred.

In addition to the central nervous system there was also a more pronounced accumulation in the liver of  $C^{14}$  dehydroascorbic acid than of  $C^{14}$  ascorbic acid. In the present study the uptake at 5 minutes after injection of  $C^{14}$  dehydroascorbic acid was twice that noted after  $C^{14}$ -ascorbic acid. In the kidney the uptake was quite the contrary. The concentration at 5 minutes after injection of  $C^{14}$  ascorbic acid was twice that of  $C^{14}$  dehydroascorbic acid. The

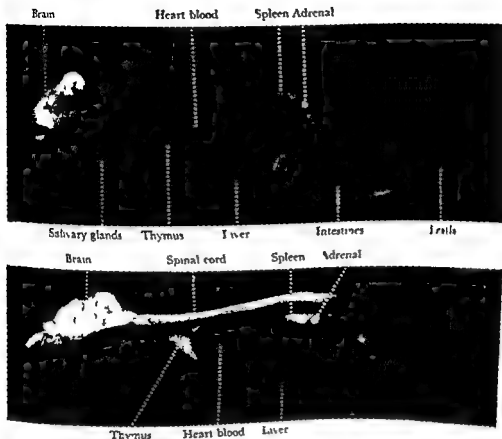


Fig. 53 Upper Autoradiogram of a mouse 4 days after an iv injection of ascorbic acid and  $C^{14}$

Fig. 54 Lower Autoradiogram of a mouse 4 days after an iv injection of ascorbic acid and  $C^{14}$

The distribution patterns are now very similar. Note the increasing uptake in the brain, adrenal and lymphatic tissues.

radioactivity left both the liver and the kidney more  $C^{14}$  than most other tissues. The differences in uptake as well as the  $C^{14}$  distribution are in agreement with the autoradiographic investigations.

The rather complicated curve of  $C^{14}$  ascorbic acid in the sublingual gland may be put in relation to the autoradiographic findings. The sublingual gland rapidly showed a marked concentration of  $C^{14}$  and  $C^{14}$ . The radioactivity in this gland then seemed to decrease rapidly, while there was a slow uptake in the sublingual gland which was  $C^{14}$  and  $C^{14}$  after injection. This seems to be in good agreement with the diagram.

The uptake in the spleen seemed to proceed with  $C^{14}$  and  $C^{14}$  of which one of the two forms that had been injected.



To summarize some of the differences in rate of uptake of ascorbic acid 1-C<sup>14</sup> and dehydroascorbic acid 1-C<sup>14</sup> by the various tissues noted in the autoradiographic investigation and at the impulse counting Ascorbic acid 1 C<sup>14</sup> was more rapidly taken up in the adrenal cortex kidneys intestinal mucosa and in the developing enamel Dehydroascorbic acid 1 C<sup>14</sup> was more rapidly accumulated in the central nervous system the liver and in the pancreatic islets of adult mice It should however be noted that neither C<sup>14</sup> dehydroascorbic acid nor C<sup>14</sup> ascorbic acid were specifically accumulated in the pancreatic islets of young animals

The great differences seem to occur only after intravenous injection as indicated by the distribution pattern of intraperitoneally injected dehydroascorbic acid 1-C<sup>14</sup>

## DISCUSSION

The chromatographic investigation indicated that after injection of ascorbic acid  $1\text{ C}^{14}$  the radioactivity remaining in the body mainly represented non metabolized ascorbic acid and labelled degradation products were evidently rapidly excreted which is in agreement with earlier data (Burns et al 1951 Curtin and King 1955). Also after an intravenous injection of dehydroascorbic acid  $1\text{ C}^{14}$  the radioactivity in the tissues almost exclusively seemed to represent ascorbic acid. This is in accordance with the results of Dayton et al (1966) who analysed the liver spleen, adrenals testes and kidneys of guinea pigs and rats 24 hours after oral or intraperitoneal administration of dehydroascorbic acid  $1\text{ C}^{14}$  and found at least 95 per cent of the radioactivity in these tissues to represent ascorbic acid.

The chromatographic results are also in agreement with the findings by Patterson and Mastin (1951) who found a rapid increase of the reduced ascorbic acid concentration in the brain and eye after intravenous injection of dehydroascorbic acid.

The reduction seemed to occur very rapidly in the brain liver kidney and submaxillary salivary glands. It is however uncertain whether dehydroascorbic acid was also reduced by the adrenal cortex and spleen since the accumulation of radioactivity in these tissues was slow and the accumulated ascorbic acid  $1\text{ C}^{14}$  might have been reduced in some other tissues. *In vitro* studies have shown that dehydroascorbic acid is reduced by the kidney liver and intestinal mucosa of rats and by the liver muscle small intestine and erythrocytes of guinea pigs (Borsook et al 1937 Schultze et al 1938).

The ratio between the concentrations of dehydroascorbic acid and ascorbic acid which was found after an intravenous injection of any one of the two substances agrees well with the concentration ratios in the tissues of guinea pigs reported by Damron et al (1952). Still it is uncertain however whether these small amounts of dehydroascorbic acid actually are present in the tissues or they are formed during the analytical procedure.

In the chromatographic investigations of the tissue extracts up to 10 per cent of the spotted radioactivity remained at the origin and might represent oxalic acid. This seems however unlikely since this amount remained at the origin also when the injection solutions of ascorbic acid  $1\text{ C}^{14}$  and dehydroascorbic acid  $1\text{ C}^{14}$  were chromatographed and in the animals there was no indication of a distribution pattern similar to that seen after injection of  $\text{C}^{14}$  oxalic acid (Fig 55) (Hammarstrom unpublished).

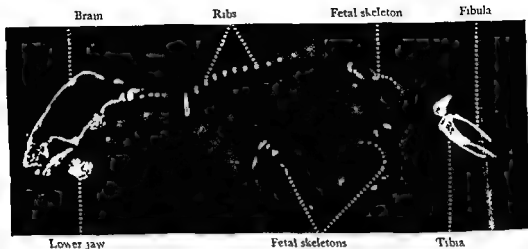


Fig 55 Autoradiogram of a pregnant mouse 4 hours after an iv injection of  $C^{14}$  oxalic acid. There is a high concentration (light areas) both in the maternal and fetal skeletons. In the other tissues the radioactivity is hardly visible (Hammarstrom unpublished)

After an intraperitoneal injection of ascorbic acid-1  $C^{14}$  the autoradiographic distribution pattern was very similar to that seen after an intravenous injection. When dehydroascorbic acid 1  $C^{14}$  was given intraperitoneally however the distribution pattern was very different from that seen after the intravenous injections. Part of the administered dehydroascorbic acid seems to have been reduced to ascorbic acid during the resorption as indicated by the slow increase of radioactivity in the brain. This is consistent with the fact that all the pharmacological effects of dehydroascorbic acid have been noted after injection of dehydroascorbic acid directly into the blood (Patterson 1949, 1950; Patterson and Mastin 1951; Wegmann 1956; Lyns and Tegeler 1963). A degradation of some of the labelled dehydroascorbic acid seems also to have occurred possibly to  $C^{14}$  oxalic acid as indicated by the high accumulation in bone. Considerably more urinary  $C^{14}$  oxalate has been found after an intraperitoneal injection of dehydroascorbic acid 1  $C^{14}$  than of ascorbic acid 1  $C^{14}$  (Curtin and King 1955; Dayton et al 1959). This may indicate that dehydroascorbic acid is not reduced in the peritoneal fluid. It has earlier been shown that dehydroascorbic acid is not reduced in the blood plasma nor in the cerebrospinal fluid (Plaut and Bulow 1935; Borsook et al 1937; Schultze et al 1938).

Dehydroascorbic acid is considered to be more lipid soluble than ascorbic acid and has been suggested to be the form for transport across cellular membranes (Patterson and Mastin 1951). The oxidation of ascorbic acid to dehydroascorbic acid was suggested to occur in the kidney and possibly in the intestines (Martin 1961; Martin and Mecca 1961).

The distribution patterns of the two vitamin forms give little if any support

to these theories. As a matter of fact the radioactivity seemed to leave the circulating blood more slowly after an injection of dehydroascorbic acid  $1\text{ C}^{14}$  than after ascorbic acid  $1\text{ C}^{14}$ .

The labelled dehydroascorbic acid apparently entered the brain directly from the blood vessels whereas there seemed to be a complete blood brain barrier to the reduced form. If ascorbic acid had been oxidized somewhere else in the body for the transport into the brain there should have been some penetration of the radioactivity directly from the blood vessels after injection of  $1\text{ C}^{14}$  ascorbic acid. This does not exclude the possibility that ascorbic acid is oxidized to dehydroascorbic acid in the choroid plexa or in the cerebrospinal fluid for the penetration into the brain. According to Plaut and Bülow (1935) however ascorbic acid occurs entirely in the reduced form in the cerebrospinal fluid in man and rabbit.

The adrenal cortex was not able to accumulate dehydroascorbic acid whereas labelled ascorbic acid was rapidly taken up there. This difference in accumulation makes it less probable that dehydroascorbic acid is the physiological form for transportation of vitamin C into the cells of the adrenal cortex.

In several other tissues the rate of uptake seemed to be independent of which one of the two forms that was administered. In these tissues—e.g. lymphoid tissues, ganglia, testes—there was no indication whether it was dehydroascorbic acid or ascorbic acid that actually entered the cells. The placental transfer of radioactivity was slow independently of which one of the two vitamin forms that had been injected and the distribution pattern in the fetal tissues was similar to that seen in the adult animals after injection of ascorbic acid  $1\text{ C}^{14}$ . This may indicate that ascorbic acid is the form for the placental transfer which also argues against the theory of Martin and Mecca (1961).

The results of the autoradiographic investigation seem to be in accordance with most earlier histochemical studies on the localization of vitamin C, reviewed by Clara (1954). The histochemical localization of ascorbic acid in the central and peripheral nervous system, adrenal cortex and medulla, ovary, testis, pancreatic islets were also observed in the present autoradiographic investigation. Also in cartilage, bone and dentin the positive histochemical reactions seem to have been specific for vitamin C (Bourne 1956, Naujoks 1956, Harndt 1966). In some investigations by Clara (1954) and others a strong positive histochemical reaction has been obtained in the capsule of the adrenal and in the tissues surrounding the lymph nodes of the gastrointestinal tract. There was no indication of such a distribution in the present investigation and it indicates that some diffusion may occur in the  $\text{C}^{14}$  nitrate technique. A comparison of histochemical and autoradiographic results will be made in the discussion of the distribution in the various systems.

Both ascorbic acid and dehydroascorbic acid have been shown to take part in the synthesis of some biogenic amines. For the hydroxylation of tryptophan to 5-hydroxytryptophan—the precursor of serotonin—dehydroascorbic acid is probably required. The possible mechanism of this hydroxylation is that a hydride ion in the 5 position of the tryptophan ring is replaced by a hydroxyl ion from the water. The resulting two protons and two electrons then probably reduce dehydroascorbic acid to ascorbic acid (Cooper, 1961; Cooper and Melcer, 1961). Hydroxylation of tryptophan to 5-hydroxytryptophan has been demonstrated *in vitro* in the intestinal mucosa, liver and to a small extent in the kidney (Cooper, 1961; Cooper and Melcer, 1961; Freedland et al., 1961). It has also been demonstrated in the mammalian brain both *in vivo* and *in vitro* (Gal et al., 1963; Grahame-Smith, 1964; Consolo et al., 1965; Green and Sawyer, 1966).

Ascorbic acid has been shown to be a co-factor at the hydroxylation of dopamine to norepinephrine. This reaction requires molecular oxygen and ascorbic acid, which functions as an electron donor, is oxidized to dehydroascorbic acid (Levin et al., 1960; Kaufman and Friedman, 1965; Kaufman, 1966).

It is interesting to note that several tissues where dehydroascorbic acid and ascorbic acid were accumulated are probable sites of synthesis of these amines. For example, the localization in the central nervous system and in the ganglia may be put in relation to these functions. In the endocrine organs, labelled 3,4-dihydroxyphenylalanine has been found to accumulate in the parathyroid, the parafollicular cells of the thyroid, the adrenal medulla and in some few cells in the pancreatic islets, possibly the A cells (Hammarstrom, Johansson, Ullberg, in preparation), which are all tissues where ascorbic acid- $C^{14}$  seemed to be taken up. Labelled 5-hydroxytryptophan is taken up in the adrenal medulla, the parafollicular cells of the thyroid and in the B cells of the pancreatic islets (Ritzen et al., 1965), where also dehydroascorbic acid was rapidly accumulated.

The main function of vitamin C in the metabolism of collagen seems to be a co-factor in the hydroxylation of proline to hydroxyproline and possibly also of lysine to hydroxylysine (Gould and Woessner, 1957; Robertson, 1964; Udenfriend, 1966). Like the  $\beta$ -hydroxylation of dopamine, also these reactions require molecular oxygen and ascorbic acid is the form needed (Fujimoto and Tamura, 1962; Prockop et al., 1962).

In the present investigation there was a low concentration of radioactivity in the proper connective tissue. This type of collagen is little sensitive to ascorbic acid deficiency (Gould, 1961). It is probably due to the slow rate of metabolism (Neuberger and Slack, 1953; Robertson, 1961). In the hard tissues of the young animals, however, a higher concentration of  $C^{14}$ -ascorbic acid was seen rapidly after injection. This may be put in relation to the

synthesis of hydroxyproline and hydroxylysine, which suggests these reactions to be directly implicated in the mineralization process

It may be of interest to correlate more in detail the distribution patterns of ascorbic acid-1  $C^{14}$  and dehydroascorbic acid 1- $C^{14}$  and their demonstrated biochemical functions with the various symptoms of a deficiency of vitamin C

### *The nervous system*

Rather little work has been done on the physiological relations between ascorbic acid and the central nervous system. Both a decreased and an increased concentration seem to affect the normal function of the brain. In experimental ascorbic acid deficiency in man Crandon et al (1940) noted an extreme fatigue as an early sign of scurvy. Monkeys fed a diet deficient in vitamin C became debile (Shaw 1949). Also in guinea pigs an impaired nervous activity has been noted (Follis 1958).

If the concentration of ascorbic acid in the brain is increased by injection of dehydroascorbic acid an intense hyperactivity of short duration has been noted (Patterson and Mastin 1951) and also a rise of the higher nervous activity lasting for several days (Lyhs and Tegeler 1963). The relation between the deficiency symptoms, the effects of an increased concentration of ascorbic acid and the synthesis of serotonin and catecholamines in the brain is not known.

Most of the monoamine containing cells in the brain seem to be localized in the brain stem (Vogt 1954, Bertler and Rosengren 1959, Carlsson et al 1962, Dahlström and Fuxe 1965).

The whole rat brain has been found to contain appreciable amounts of dopamine  $\beta$  hydroxylase *in vitro*. The highest enzymic activity seems to be in hypothalamus and caudate nucleus, little in cerebellum and no detectable activity in cortex (Udenfriend and Creveling 1959). Masuoka et al (1963) found tyrosine to be converted to norepinephrine by cat brain slices from hypothalamus, visual cortex and reticular area but not from caudate nucleus.

The bulk of the tryptophan hydroxylase activity in the brain seems to be localized in the brain stem as indicated by *in vitro* studies on rat brain homogenates. No activity could be demonstrated in cerebellum (Green and Sawyer 1966).

When the  $C^{14}$  labelled precursors 3,4 dihydroxyphenylalanine or 5 hydroxy tryptophan were injected there was a rather even distribution of radio activity in almost all the brain (Rosell et al 1963, Ruzan et al 1965). Both the substances showed a higher concentration in the choroid plexa and in the pineal body than elsewhere in the brain (Ruzan et al 1965, Hammarstrom, Johansson, Ullberg in preparation).

According to the autoradiograms ascorbic acid was accumulated in almost all the brain tissue but the highest concentrations seemed to be in the hippo

campus and the cerebellar cortex Apparently there is little agreement with the areas where the main synthesis and localization of biogenic monoamines have been demonstrated and the localization of the highest concentration of ascorbic acid

Histochemical studies with the acid silver nitrate technique of Giroud and Leblond (1934) indicate that all the nerve cells of the central nervous system contain ascorbic acid (Clara 1954) The positive reaction was obtained in the cell bodies and only occasionally in the dendrites There were great variations in the concentration of ascorbic acid between the various cell types and also between the cells of the same type Strong positive reactions were obtained in cells in the cerebral cortex striatum thalamus cerebellar cortex and in certain cells in other parts of the brain In the vegetative nuclei there were cells with a strong positive reaction (Clara 1954) The results of these histochemical studies seem to be in agreement with the autoradiographic distribution pictures However a more detailed autoradiographic investigation is necessary if the localization and concentration of ascorbic acid in all the indicated areas are to be discussed

The mode of uptake of ascorbic acid in the brain is of special interest since there appeared to be a complete blood brain barrier to the reduced form whereas the oxidized form easily passed directly from the blood vessels into the brain The existence and localization of a blood brain barrier has caused considerable debate (*cf* Dobbing 1961) The localization of the barrier for ascorbic acid seemed to be the same as that observed for trypan blue (King 1939 Wislocki and Leduc 1952) The pituitary the pineal body and the choroid plexa are outside the barrier and they rapidly accumulated the radioactivity of ascorbic acid  $1\text{ C}^{14}$  There seems to be a similar blood brain barrier to the catecholamines and serotonin (Bertler et al 1966 Hamberger and Hamberger 1966)

From the choroid plexa the radioactivity of  $\text{C}^{14}$ -ascorbic acid seemed to enter the brain possibly together with simultaneously secreted cerebrospinal fluid and then proceed *per continuitatem* into the brain The concentration of ascorbic acid in the cerebrospinal fluid of rabbits is almost twice that of the blood plasma (Plaut and Bulow 1935)

There seemed to be a similar barrier to the penetration of ascorbic acid into the eye and there was some latency before the radioactivity of  $\text{C}^{14}$  ascorbic acid was visible in the retina It is interesting to note that ascorbic acid has been shown to enter the eye as a result of a secretory process and seems to be involved in the secretion of the intraocular fluid (Friedenwald et al 1943 Kinsey 1950)

Recently adrenergic structures have been demonstrated in three zones in the inner plexiform layer of the retina of mice and several other species (Malmfors 1963 Häggendahl and Malmfors 1963 1965 Ehinger 1966) This

may be correlated to the marked uptake of ascorbic acid in the ganglion cell layer and the inner nuclear layer

In the peripheral nervous system the radioactivity of labelled ascorbic acid as well as dehydroascorbic acid was taken up in significant amounts in the ganglia and the highest concentration was found in the sympathetic ganglia. This is consistent with the histochemical results in humans where a substance capable of reducing acid silver nitrate was found in almost all the nerve cells of the sympathetic ganglia and in some few cells in the sensory ganglia. In rats it is necessary to give ascorbic acid parenterally for the demonstration of the localization in the spinal ganglia (cf. Clara 1954).

Catecholamines mainly norepinephrine have been demonstrated in the autonomic ganglia but so far not in the sensory ganglia. After injection of  $C^{14}$ -labelled 3,4-dihydroxyphenylalanine however there was not only a marked uptake of radioactivity in the sympathetic ganglia but also a moderate accumulation in the spinal ganglia (Hammarström, Johansson, Ullberg in preparation).

In the ganglia of Auerbach's plexus in the gastrointestinal walls both the reduced and oxidized ascorbic acid was rapidly accumulated. These ganglia have been shown by autoradiography to accumulate 3,4-dihydroxyphenylalanine  $H^3$  and 5-hydroxytryptophan  $H^3$  (Gershon et al. 1965; Hammarström et al. 1966; Hammarström, Johansson, Ullberg in preparation). Adrenergic structures in these ganglia have also been demonstrated by fluorescence microscopy (Norberg 1964; Hollands and Vanov 1965).

### *The circulatory system*

Petechial hemorrhage is a prominent symptom of ascorbic acid deficiency. Little is known about the biochemical mechanism behind this defect and it is generally explained by an assumed lack of an endothelial cement substance. Injection of a dye to vitamin C deficient guinea pigs indicates however that there is no increased vascular permeability (Flister and Schack 1950). Recent investigations have shown that ascorbic acid is probably involved in the maintenance of the tone of the blood vessels (Lee 1961). This is consistent with the present investigation since no specific uptake of the reduced or oxidized form of ascorbic acid was demonstrable in the walls of the blood vessels whereas a considerable concentration was noted in the sympathetic ganglia. The temporarily increased blood pressure after injection of dehydroascorbic acid may also be noted in this connection (Patterson and Mastin 1951; Wegmann 1958).

The blood platelets contain large amounts of serotonin and the possible role of dehydroascorbic acid in the synthesis of this amine may also be related to the scorbutic hemorrhages. Serotonin has been claimed to increase the capillary resistance both in man and experimental animals and to reduce



the bleeding time. The results in this field are however contradictory (cf Erspamer 1961).

In the present investigation the radioactivity seemed to persist longer in the blood after injection of dehydroascorbic acid  $1\text{ C}^{14}$  than after ascorbic acid  $1\text{ C}^{14}$ . This may be put in relation to the penetration of dehydroascorbic acid into erythrocytes *in vitro* which has been noted (Lloyd and Sinclair 1953).

The concentration of ascorbic acid in leucocytes and blood platelets has been reported to be 20—40 times that of the blood plasma (cf Lloyd and Sinclair 1953). The relatively high concentration of radioactivity in the lymphatic tissues which was noted in the present investigation seems to be consistent with these data. In scurvy the phagocytosis of the mononuclear leucocytes is considered to be impaired (Follis 1958). Leblond (1934) has demonstrated a positive histochemical reaction for vitamin C in the mononuclear leucocytes.

The uptake in the bone marrow may be put in relation to the anemia associated with ascorbic acid deficiency (cf Lloyd and Sinclair 1953, Degkwitz et al 1965).

### *The digestive system*

The high concentration of ascorbic acid in the tissues of the alimentary tract does not seem to be related to a large excretion. The concentration of ascorbic acid in the saliva has been found to be very low. In humans it seems to be 5—10 times less than the concentration in the blood. In the gastric secretion the concentration is about the same as in the blood (cf Lloyd and Sinclair 1953). The fecal excretion of ascorbic acid is very low and after an intraperitoneal injection of ascorbic acid  $1\text{ C}^{14}$  to guinea pigs less than 1 per cent of the dose appeared in the faeces during 24 hours (Burns et al 1951).

After injection of labelled 3,4 dihydroxyphenylalanine to mice there was a considerable accumulation of radioactivity in the submaxillary salivary gland (Rosell et al 1963). The accumulation of labelled 5 hydroxytryptophan seems to be less pronounced (Ritzen et al 1965). By fluorescence microscopy has been shown that the submaxillary gland in the rat is richly supplied with adrenergic structures whereas the sublingual salivary gland seems to be devoid of adrenergic innervation (Norberg and Hamberger 1964, Norberg and Olson 1965).

The function of ascorbic acid in the gastro intestinal mucosa does not seem to be known. Some of the radioactivity may be put in relation to the synthesis of serotonin in the enterochromaffin cells. The distribution of radioactivity however was rather even and no specific uptake in these cells could be observed.

A positive histochemical reaction for vitamin C has been demonstrated in most cells of the mucosa of the human stomach and small intestine. Especially strong reaction has been obtained in the enterochromaffin cells (Clara 1943). In guinea pigs and rats these cells seem to contain less ascorbic acid as indicated by the histochemical studies (Pfuhl 1941 Clara 1954). In agreement with the present investigation the concentration of ascorbic acid in the cells of the mucosa of the large intestine has been found to be less than in the mucosa of the stomach and small intestine.

#### *The respiratory system*

A rather strong accumulation of radioactivity was observed in the bronchial and tracheal epithelium and also in the nasal mucosa. The localization may be put in relation to the monoamine containing cells in the tracheal epithelium which has recently been demonstrated (Larson et al 1966).

#### *The endocrine system*

In ascorbic acid deficiency the adrenal gland has been found to hypertrophy and there is also a decrease in the concentration of cholesterol in the adrenal (Banerjee and Deb 1951 Eisenstein and Shank 1951). Stimulation of the adrenal gland with ACTH causes a decreased concentration of both ascorbic acid and cholesterol (Sayers et al 1944). Since cholesterol is a precursor to steroid hormones a role for ascorbic acid in the synthesis of some of these has been suggested. Several investigations indicate that ascorbic acid is concerned with the hydroxylation of some precursors to steroid hormones (cf Degkwitz et al 1965). So far no definite function has been established for ascorbic acid in the adrenal cortex.

There are several theories for the explanation of the decrease in ascorbic acid in the adrenal cortex during stimulation (cf Pirani 1952 Lloyd and Sinclair 1953 Degkwitz et al 1965). Ascorbic acid does not seem to be metabolized by the adrenal to substances no longer identifiable as ascorbic acid or dehydroascorbic acid (Salomon 1952).

In the present investigation dehydroascorbic acid  $C^{14}$  had almost no affinity to the adrenal cortex. It is possible that ascorbic acid is oxidized at the reactions induced by ACTH in the adrenal cortex and then leaves the cells as dehydroascorbic acid. Dehydroascorbic acid has been shown to be rapidly taken up and reduced by the erythrocytes (Lloyd and Sinclair 1953). This may explain why Vogt (1948) found no increase in total ascorbic acid (ascorbic acid + dehydroascorbic acid) in venous blood plasma from the adrenal of dogs and cats after treatment with ACTH. This is also consistent with the results of Slusher and Roberts (1957) who noted an increase in the total ascorbic acid in the whole blood from the adrenal after ACTH stimulation.

According to earlier histochemical investigations the concentration of ascorbic acid in the adrenal cortex is high in both the zonae fasciculata and reticularis but lower in the zona glomerulosa (Leblond 1934 Clara 1954) This is in agreement with the distribution of radioactivity 4 hours after injection of ascorbic acid-1- $C^{14}$  and dehydroascorbic acid-1- $C^{14}$  Shortly after injection of ascorbic acid 1  $C^{14}$  however the highest concentration in the adrenal cortex was found in the zona glomerulosa

In the adrenal medulla an even distribution of ascorbic acid has been demonstrated histochemically There has been great variations in the strength of the positive reactions and there has been doubt whether these are true reactions of vitamin C (Clara 1954) Both ascorbic acid and dehydroascorbic acid may be concerned with the synthesis of catecholamines and serotonin in the adrenal medulla Serotonin has recently been shown to be a physiological constituent of the adrenal medulla (Snyder et al 1965)

The concentration of ascorbic acid in the ovary is influenced by hormonal stimulation like that of the adrenal cortex (Claesson et al 1949 Hokfelt 1950) Also in the ovary ascorbic acid has been suggested to take part in the synthesis of steroid hormones It is therefore remarkable that there was not the same difference in the accumulation rate after injection of the labelled vitamin forms as was observed in the adrenal cortex The fact that there was a rather slow uptake of radioactivity after injection of either one of the two vitamin forms may be related to the advanced stage of pregnancy of the animals It has been shown that the corpora lutea contain considerably less ascorbic acid in late pregnancy than in the earlier stages (Giroud and Ratsimamanga 1942)

The distribution of radioactivity in the ovary showed great similarities with the distribution of injected  $C^{14}$  cholesterol (Appelgren in preparation)

Ascorbic acid seems to be of great importance for the carbohydrate metabolism Scorbutic guinea pigs are reported to have a reduced glucose tolerance (Sigal and King 1936) Their pancreatic islets are increased in number and size according to Banerjee (1944) but the cells contain few granules There is a decreased amount of insulin in the pancreas and a lowered deposition of liver glycogen (Banerjee and Ghosh 1947) Injection of dehydroascorbic acid induced a temporary or permanent diabetes (Patterson 1949 1950)

In the present investigation it is interesting to note the rapid uptake of radioactivity in pancreatic islets after injection of dehydroascorbic acid 1  $C^{14}$  and the more slow accumulation of ascorbic acid 1  $C^{14}$  Since the B cells constitute the major part of the pancreatic islet the autoradiographic distribution pictures indicate an accumulation at least in these cells An uptake also in other islet cells seems probable

Serotonin has been demonstrated in the pancreatic B cells of guinea pigs and mice (Falk and Hellman 1963 1964 Ritzén et al 1965) A metastasizing

B cell adenoma in man has also been described which had an excessive production of both insulin and serotonin (Gloor et al 1964). In the duck a primary catecholamine has been demonstrated in the A cells (Cegrell et al 1964). After injection of  $H^3$ -labelled 3,4 dihydroxyphenylalanine to mice and rats there was a marked uptake of radioactivity in some few cells of the pancreatic islet possibly the A cells (Hempel 1963 Hammarström Johansson Ullberg in preparation).

The rapid uptake of dehydroascorbic acid  $1-C^{14}$  and the presence of serotonin in the B cells of the pancreatic islet makes it likely that dehydroascorbic acid is involved in the synthesis of serotonin also in these cells. This may suggest a role for serotonin in the induction of diabetes by dehydroascorbic acid. Also the normal function of vitamin C in the synthesis and/or release of insulin may be mediated via dehydroascorbic acid and serotonin. Ascorbic acid may also be implicated in the metabolism of the catecholamine containing cells of the pancreatic islets.

In most species investigated histochemically there was a negative reaction for vitamin C unless parenteral administration of ascorbic acid preceded the investigation. After administration of ascorbic acid almost all the cells of the pancreatic islet showed a positive reaction. Some few cells possibly the A cells seemed to contain more ascorbic acid than the average (cf Clara 1954).

Alloxan which is chemically very similar to dehydroascorbic acid has recently been shown to be specifically taken up in the pancreatic islets of adult mice but not in the islets of young mice and rats (Hammarström and Ullberg 1966 Hammarström Hellman and Ullberg in preparation). Young animals have been found to be resistant to the diabetogenic action of alloxan (Ferner 1952). It is interesting to note that neither ascorbic acid dehydroascorbic acid nor alloxan were specifically taken up in the pancreatic islets of young animals.

In the thyroid the highest concentration was found in some few scattered cells probably the parafollicular cells. These cells have been shown to contain serotonin (Falk et al 1964 Ritzén et al 1965) and possibly also a catecholamine (Larson et al 1966 Hammarström Johansson Ullberg in preparation). The function of these cells does not seem to be clarified. There is some histochemical evidence that calcitonin is synthesized there (Foster et al 1964). By means of an antibody fluorescence technique however calcitonin has been demonstrated in the ordinary follicular cell of the thyroid (Hargis et al 1966).

The parathyroid accumulated radioactivity. The function of ascorbic acid in this gland is not known but it may be put in relation to the accumulation of 3,4 dihydroxyphenylalanine  $H^3$  (Hammarström Johansson Ullberg in preparation).

### *Connective tissue cartilage bone and teeth*

The morphological changes associated with scurvy in connective tissue proper cartilage bone and teeth have been described in detail (*cf* Leicester 1949 Follis 1958) The basic biochemical defect in these tissues seems to be an impaired hydroxylation of proline and possibly also of lysine (Robertson 1964) In the present investigation there was a low concentration of radioactivity in the connective tissue proper This may be correlated to the slow rate of metabolism of this tissue and its low sensitivity to ascorbic acid deficiency (Neuberger and Slack 1953 Gould 1961 Robertson 1961)

In the odontoblasts there was a considerable uptake of radioactivity which seems to be in agreement with the fact that these cells are more sensitive to ascorbic acid deficiency than most other cells (Höjer 1926 Kautner et al 1944)

The odontoblasts fibroblasts and osteoblasts are considered to revert to an immature state in ascorbic acid deficiency (MacLean et al 1939 Thorell and Wilton 1945) The mechanism behind this maintenance of maturity by ascorbic acid seems to be obscure A considerable synthesis of collagen is going on in the odontoblasts as indicated by their uptake of labelled proline (Greulich and Slavkin 1965) Also in the other cells of the pulp the concentration of  $C^{14}$  ascorbic acid was higher than in the circulating blood This uptake does not seem to be due to an uptake in the walls of the blood vessel since no specific uptake in these could be observed elsewhere in the body In ascorbic acid deficiency the dental pulp becomes hemorrhagic and a deposition of an amorphous calcified material occurs (Höjer and Westin 1925 Boyle et al 1940)

In cartilage and bone of the young animals the concentration of  $C^{14}$  ascorbic acid was higher than in the proper connective tissue The autoradiographic localization of ascorbic acid in these tissues is in agreement with earlier histochemical studies showing a markedly positive reaction in the zone of cartilage cell division and a negative reaction in the hypertrophied cartilage cells In the calcifying cartilage and in the newly formed bone trabeculae the reaction was positive again (Bourne 1956) Also the uptake at the predentin dentin junction has earlier been demonstrated with histochemical methods (Naujoks 1956 Harndt 1966) The persisting localization of ascorbic acid in these areas may indicate a role in the physiological mineralization process In scurvy the calcified cartilage in the epiphyseal plate is claimed not to be replaced by bone trabeculae and the predentin becomes hypercalcified (*cf* Leicester 1949 Follis 1958)

In the enamel of some teeth there was a rapid and pronounced uptake of  $C^{14}$  ascorbic acid Also here the radioactivity was localized in areas where mineralization apparently was taking place as indicated by the distribution of injected radiocalcium in the teeth of rats of the same age (Fig 56) With

Uptake of  $\text{Ca}^{45}$  through  
the whole thickness of  
the enamel



Tall ameloblasts Uptake  
of  $\text{Ca}^{45}$  in a superficial  
zone of the enamel

Fig 56 Microautoradiogram (section + autoradiogram) of the first upper molar of a 10 days old rat 30 minutes after an i.p. injection of  $\text{Ca}^{45}\text{Cl}$ . There is a high concentration (black grains) throughout the thickness of the enamel at the tip of the cusps and a less marked uptake in the superficial parts of the enamel matrix underneath the tall ameloblasts in the cervical parts of the tooth. There is also an accumulation in the dentin close to the pulp. There seems to be no radioactivity on the dentin side of the amelodentinal junction in the zones of secondary mineralization of the enamel as was seen after injection of ascorbic acid  $\text{C}^{14}$  (Hammarstrom, unpublished) ( $\times 40$ )

time the  $\text{C}^{14}$  ascorbic acid seemed to be redistributed to areas where mineralization was going on at the moment

Ascorbic acid deficiency is considered to have no direct effect upon enamel formation (Boyle 1938 Wassermann 1944 Iullmer et al 1961). The dental changes associated with scurvy however have mainly been studied using decalcified histological sections and possible changes in the composition might not have been detected

It has been shown that during the mineralization of the enamel there is a considerable loss of organic material of the matrix once deposited. There is at least an 80 per cent (w/v) decrease in the concentration of all amino acids except hydroxyproline and hydroxylysine (Deakins 1942 Weinmann

et al 1942 Eastoe 1963 Burgess and Maclaren 1965) In fact there seem to be some increase in the amount of hydroxyproline (Burgess and Maclaren 1965) The major changes in the composition of the organic components of the enamel matrix apparently take place immediately after the matrix reaching its final thickness The rate of development seems to be identical with the one of rapid mineralization and uptake of ascorbic acid No significant amounts of hydroxyproline and hydroxylysine have been demonstrated in fetal enamel (Eastoe 1960 Glimcher et al 1961 Piez 1961) In mature enamel on the other hand several investigators have demonstrated both hydroxyproline and hydroxylysine (Hess et al 1953 Stack 1954 Battistone and Burnett 1956 Burgess and Maclaren 1965) The amounts are small and contamination may easily occur both from the dentin and from the saliva and a possible coronal cementum (Glimcher et al 1964) It is however tempting to assume that ascorbic acid is involved in the hydroxylation of proline and lysine also in the enamel The fact that only the reduced form was accumulated in the enamel matrix support this suggestion The hydroxylation may be of importance for a physiological deposition of minerals During scurvy amorphous calcification occurs in various tissues (Bourne 1956)

There are some reports of an impaired calcium metabolism in scurvy But the changes have been considered to be secondary to an imperfect maturation of bone and dentin (Bourne 1943 1956)

The finding by Loverud (1923) of a marked decrease in total ash and calcium in teeth of scorbutic guinea pigs is interesting in view of the possible role of ascorbic acid in the mineralization process Friberg (1958) noted decreased uptake of labelled phosphate in bone and teeth of scorbutic guinea pigs

The autoradiographic pictures of  $\text{Ca}^{45}$  and  $\text{C}^{14}$ -ascorbic acid in the enamel support the idea of a second mineralization starting in the cusps when the partly mineralized matrix has reached its final thickness (Diamond and Weismann 1940 Glock et al 1942 and others)

The autoradiographic blackening in the dentin close to the amelodentin junction apparently has no corresponding accumulation of minerals (Fig 1) Presently no specific chemical reaction is known to take place in those areas of the dentin in which ascorbic acid might take part But it indicates a possible biochemical interrelationship between reactions in the enamel and dentin of the developing teeth

The distribution of radioactivity in the periodontal tissues may indicate that the loosening of teeth in scurvy is more a matter of decreased fixation of the periodontal fibers in the bone than an impaired maintenance of the fibers themselves This is in agreement with earlier investigations showing a marked resorption of the alveolar bone associated with ascorbic acid deficiency (Wagner 1932 Boyle 1941 Shaw et al 1945)

## SUMMARY

Ascorbic acid  $C^{14}$  and dehydroascorbic acid  $C^{14}$  have been administered intravenously to adult male mice pregnant female mice and 16 days old rats and intraperitoneally to 10 days old rats. The distribution of the injected substances at various intervals (2 minutes—6 days) after injection has been studied by whole body autoradiography microautoradiography and impulse counting. The chemical identity of the radioactivity in the tissues at various intervals (5 minutes—4 days) after injection has been investigated by thin layer chromatography of extracts of some selected tissues which showed a high concentration of radioactivity.

The chromatographic investigation indicated that the radioactivity in the adrenals liver spleen kidney salivary glands and brain almost exclusively represented ascorbic acid regardless of whether ascorbic acid  $C^{14}$  or dehydroascorbic acid  $C^{14}$  had been injected. In spite of the rapid reduction of injected  $C^{14}$  dehydroascorbic acid the distribution of the two vitamin forms were very different after intravenous injection. The distribution patterns gradually became similar but as long as 3—4 days after injection some differences were noticeable. The distribution patterns have been described and discussed in detail. The results which functionally seem to be most interesting have been summarized below.

After an intravenous injection of  $C^{14}$ -ascorbic acid the accumulation in the central nervous system proceeded very slowly. There seemed to be a complete blood brain barrier and the radioactivity penetrated from the choroid plexa and from the periphery possibly together with simultaneously secreted cerebrospinal fluid. At 1 day after injection the central nervous system showed the highest concentration in the body.

After an intravenous injection of  $C^{14}$  dehydroascorbic acid the concentration in the central nervous system rapidly increased and as early as 2 minutes after injection radioactivity was found in the whole brain and spinal cord and the concentration markedly exceeded that in the blood. The striking difference in rate of penetration was also demonstrated by the impulse counting investigation. After as long as 4 days the concentration in the brain was higher after injection of  $C^{14}$  dehydroascorbic acid than after  $C^{14}$  ascorbic acid.

Injection of dehydroascorbic acid has been found to cause an intense hyperactivity of short duration (Patterson and Austin 1951) and also a rise in the higher nervous activity lasting for several days (Lyhs and Tegeler 1963). This may be put in relation to the rapid and persistent accumulation of



et al 1942 Eastoe 1963 Burgess and Maclaren 1965) In fact there seems to be some increase in the amount of hydroxyproline (Burgess and Maclaren 1965) The major changes in the composition of the organic components of the enamel matrix apparently take place immediately after the matrix reaching its final thickness This state of development seems to be identical with the one of rapid mineralization and uptake of ascorbic acid No significant amounts of hydroxyproline and hydroxylysine have been demonstrated in fetal enamel (Eastoe 1960 Glimcher et al 1961 Picz 1961) In mature enamel on the other hand several investigators have demonstrated both hydroxyproline and hydroxylysine (Hess et al 1953 Stack 1954 Battistone and Burnett 1956 Burgess and Maclaren 1965) The amounts are small and contamination may easily occur both from the dentin and from the saliva and a possible coronal cementum (Glimcher et al 1964) It is however tempting to assume that ascorbic acid is involved in the hydroxylation of proline and lysine also in the enamel The fact that only the reduced form was accumulated in the enamel may support this suggestion The hydroxylation may be of importance for a physiological deposition minerals During scurvy an amorphous calcification occurs in various tissues (Bourne 1956)

There are some reports of an impaired calcium metabolism in scurvy But the changes have been considered to be secondary to an imperfect matrix formation of bone (Bourne 1943 1956)

The finding by (1958) of a marked decrease in total ash and calcium in teeth of ascorbic acid deficient pigs is interesting in view of the possible role of ascorbic acid in the mineralization process Friberg (1958) noted a decreased uptake of inorganic phosphate in bone and teeth of scorbutic guinea pigs

The autoradiographic studies of  $\text{Ca}^{45}$  and  $\text{C}^{14}$  ascorbic acid in the enamel support the idea of a rapid mineralization starting in the cusps when the partly mineralized matrix has reached its final thickness (Diamond and Weinmann 1940 Clock et al 1942 and others)

The autoradiographic blackening in the dentin close to the amelodentinal junction apparently has no corresponding accumulation of minerals (Fig 56) Presently no specific chemical reaction is known to take place in those areas of the dentin in which ascorbic acid might take part But it indicates a possible biochemical interrelationship between reactions in the enamel and dentin of the developing teeth

The distribution of radioactivity in the periodontal tissues may indicate that the loosening of teeth in scurvy is more a matter of decreased fixation of the periodontal fibers in the bone than an impaired maintenance of the fibers themselves This is in agreement with earlier investigations showing a marked resorption of the alveolar bone associated with ascorbic acid deficiency (Wagner 1932 Boyle 1941 Shaw et al 1945)

## ACKNOWLEDGEMENTS

My sincere thanks are due to

Associate professor Sven Ullberg for valuable discussions and stimulating criticism and for giving me the privilege of being a member of his research group His personal interest has greatly supported this work

Professor Carl G Schmiterlöw Dean of the Royal Veterinary College and Head of the Department of Pharmacology for his interest in my work and for kindly placing the facilities of the department at my disposal

Professor Yngve Ericsson Head of the Department of Cariology and the Chemical Laboratory the Dental School Karolinska Institutet for introducing me in the field of scientific research

Drs Lars Erik Appelgren Eskil Hansson and William J Waddell for stimulating discussions

Mrs Gun Mabo Mr Anders Nilsson Mrs Else Stålfelt and Mrs Ingrid Tranck for valuable technical assistance during different phases of this work

Mrs Birgitta Hagtorn for skilful preparation of the illustrations

Mrs Ulla Sunnberg for her skill and care at the preparation of the manuscripts

This investigation was supported by

Reservationsanslaget till främjande av ograduerade forskares vetenskapliga verksamhet Odont fak Karolinska Institutet

Swedish Medical Research Council grant Nos B67 24P 2117-01 and 12X 713 01

Knut & Alice Wallenbergs Stiftelse

Ragnar & Torsten Soderbergs Stiftelse

## REFERENCES

- ABT A F S von SCHUCHTIG and T FENN Vitamin C requirements of man re examined New values based on previously unrecognized exhalatory excretory pathway of ascorbic acid *Am J clin Nutr* 1963 12 21-29
- BAKER E M H E SAUBERLICH S J WOLFSKILL W T WALLACE and E E DEAN Tracer studies of vitamin C utilization in men Metabolism of d glucuronolactone 6-C<sup>14</sup> d glucuronic 6-C<sup>14</sup> acid and l ascorbic acid 1-C<sup>14</sup> *Proc Soc exp Biol Med* 1962 109 737-741
- BANERJEE S Relation of scurvy to histological changes in the pancreas *Nature* 1944 153 344-345
- BANERJEE S and C DEB Effect of scurvy on cholesterol and ascorbic acid in guinea pig adrenals *J biol Chem* 1951 190 177-180
- BANERJEE S C DEB and B BELAVADY Effect of scurvy on glutathione and dehydroascorbic acid in guinea pig tissues *J biol Chem* 1951 195 271-276
- BANERJEE S and N C GHOSH Relation of scurvy to glucose tolerance test liver glycogen and insulin content of pancreas of guinea pigs *J biol Chem* 1947 168 207-211
- BATTISTONE G C and G W BURNETT Studies on the composition of teeth IV The amino acid composition of human enamel protein *J dent Res* 1956 35 260-262
- BERTLER A B FAICK CH OWMAN and L ROSENGREN The localization of monoaminergic blood brain barrier mechanisms *Pharmac Rev* 1966 18 369-385
- BERTLER A and F ROSENGREN Occurrence and distribution of catechol amines in brain *Acta physiol scand* 1959 47 350-361
- BESSEY O A A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of other reducing substances *J biol Chem* 1938 126 771-784
- BORSOOK H H W DAVENPORT C E P JEFFREYS and R C WARNER The oxidation of ascorbic acid and its reduction in vitro and in vivo *J biol Chem* 1937 117 237-279
- BOURNE G H The staining of vitamin C in the adrenal glands *Aust J exp Biol Med* 1933 11 261-267
- The vitamin C technique as a contribution to cytology *Anat Rec* 1936 66 369-385
- Some experiments on the possible relationship between vitamin C and calcification *J Physiol (London)* 1943 102 319-328
- Vitamin C and bone In »The biochemistry and physiology of bones» Ed G H Bourne Academic Press 1956 539-574
- BOYLE P F The effect of ascorbic acid deficiency on enamel formation in the teeth of guinea pigs *Am J Path* 1938 14 843-848
- BOYLE P F Effect of various dietary deficiencies on the periodontal tissues of the guinea pig and of man *J Am dent Ass* 1941 8 1788-1793
- BOYLE P F O A BLISSAY and I R HOWE Rate of dentin formation in incisor teeth of guinea pigs on normal and ascorbic acid deficient diets *Archs Path* 1940 30 90-107
- BRUNACHER C and J P VILLERMEYR Biologische Wirksamkeit und Stoffwechsel der Ascorbinsäure und verwandter Substanzen In »Ascorbinsäure» Ed K Lang Wiss Veroff dt Ges Ernähr 1964 14 61-79
- BURGESS H C and C M MACLAREN I t in in developing bovine enamel In »Tooth enamel» Ed M A Stack and R W Fearnley Verlag W. S. N. Ltd 1965 74-82
- BURNS J J Ascorbic acid In »Vitamins» Ed F L D M Greenberg Academic Press 1960 1 341-356

- BURNS J J H B BURCH and C G KING The metabolism of  $l$ -C<sup>14</sup> ascorbic acid in guinea pigs  
J biol Chem 1951 191 501-514
- BURNS J J P G DAYTON and S SCHULENBERG Further observations on the metabolism of  $l$ -ascorbic acid in guinea pigs J biol Chem 1956 218 15-21
- BURNS J J E H MOSBACH and S SCHULENBERG Ascorbic acid synthesis in normal and drug treated rats studied with  $l$ -ascorbic  $l$ -C<sup>14</sup> acid J biol Chem 1954 207 679-687
- CARLSSON A B FALCK and V V HILLARP Cellular localization of brain monoamines Acta physiol scand 1962 36 suppl 196
- CEGRELL L B FALCK and B HELLMAN Monoaminergic mechanisms in the endocrine pancreas In «The structure and metabolism of the pancreatic islets» Ed H E Brolin B Hellman H Knutson Pergamon Press 1964 429-435
- CHAN P C R R BECKER and C G KING Metabolic products of  $l$ -ascorbic acid J biol Chem 1958 231 231-240
- CHATTERJEE J B V C KAR N C GHOSH and B C GUTTA Aspects of ascorbic acid biosynthesis in animals Ann N Y Acad Sci 1961 92 36-56
- CLAESON L V Å HILLARP B HOGBERG and B HOGFELT Changes in the ascorbic acid content in the interstitial gland of the rabbit ovary following gonadotrophic stimulation Acta endocr Copenh 1949 2 249-256
- CLARA M Histotopochemische Untersuchungen über das Vitamin C in der Schleimhaut des Magen Darm Kanals beim Menschen Z mikrosk anat Forsch 1943 54 358-395
- — Ergebnisse und Probleme des histotopochemischen  $l$ -Ascorbinsäurenachweises Vitamine und Hormone 1954 6 12-97
- CON OLO S S GARATTINI R GINELNETTI P MORSELLI and L. VALZELLI The hydroxylation of tryptophan in vivo by brain Life Sciences 1965 4 625-630
- COOPER J R The role of ascorbic acid in the oxidation of tryptophan to 5 hydroxytryptophan Ann N Y Acad Sci 1961 9 208-211
- COOPER J R and I MELZER The enzymic oxidation of tryptophan to 5 hydroxytryptophan in the biosynthesis of serotonin J Pharmac exp Ther 1961 13 265-68
- CRANDON J H C C LUND and D B DILL, Experimental human scurvy N Engl J Med 1951 223 353-369
- CURTIN C OH and C G KING The metabolism of ascorbic acid  $l$ -C<sup>14</sup> and oxalic acid- $l$ - $l$  in the rat J biol Chem 1950 216 539-548
- CZOR G Gegenwärtige Höhe der Ascorbinsäurezufuhr In «Ascorbinsäuren» Ed K Lang Wiss Veroff dt Ges Ernähr 1965 14 99-106
- DAHLSTROM A and A FURU Evidence for the existence of monoamine containing neurones in the central nervous system 1 Demonstration of monoamines in the cell bodies of brain stem neurones Acta physiol scand 1965 6 suppl 232
- DAMROW C M M M MOYER and J H ROE Metabolism of  $l$ -ascorbic acid and dihydro- $l$ -ascorbic acid and diketo- $l$ -gulonic acid in the guinea pig J biol Chem 1952 192 513-524
- DANIELLI J F Establishment of cytochemical techniques Nature 1946 157 753-757
- DAYTON P G F EISENBERG JR and J J BURNS Metabolism of C<sup>14</sup> labeled ascorbic acid and dihydro- $l$ -ascorbic acid and diketogulonic acids in guinea pigs Archs Biochem Biophys 1959 11 111-118
- DAYTON P G M M SNELL and J M PEREL Ascorbic and dehydroascorbic acids in guinea pigs and rats J Nutr 1966 87 338-344
- DEAKINS M Changes in the ash value and organic content of pig enamel during calcification J dent Res 1912 21 429-435
- DECKWITZ E W SCHNEIDER and HJ STAUDINGER Biochemie der Ascorbinsäure In «Ascorbinsäuren» Ed K Lang Wiss Veroff dt Ges Ernähr 1965 14 17-3
- DEMOLE V On the physiological action of ascorbic acid and some related compounds Biochem J 1934 28 770-773

- DIAMOND M and J P WEINMANN The enamel of human teeth Columbia University Press New York 1940
- DOBBS J The blood brain barrier *Physiol Rev* 1961 *41* 130-188
- EASTOE J E Organic matrix of tooth enamel *Nature* 1960 *187* 411-412
- — The amino acid composition of proteins from the oral tissues *Archs oral Biol* 1963 *8* 633-652
- EHINGER B Ocular and orbital vegetative nerves *Acta physiol scand* 1966 *67* suppl 268
- EISENSTEIN A B and R E SHANK Relationship of ascorbic acid to secretion of adrenocortical hormones in guinea pigs *Proc Soc exp Biol Med* 1961 *78* 619-624
- ELLER K and J A SCHACK Effect of vitamin C deficiency on the diffusion of T 1824 across the capillary wall *Am J Physiol* 1950 *161* 283-288
- ERSPAMER V Recent research in the field of 5 hydroxytryptamine and related indolealkylamines *Fortschr Arzneimittelforsch* 1961 *3* 151-367
- FALK B and B HELLMAN Evidence for the presence of biogenic amines in pancreatic islets *Experientia* 1963 *19* 139-140
- — A fluorescent reaction for monoamines in the insulin producing cells of the guinea pig *Acta endocr Copenh* 1964 *45* 133-138
- FALK B B LARSON C v MECKLENBURG E ROSENBERG and K SVENARUS On the presence of a second specific cell system in mammalian thyroid gland *Acta physiol scand* 1964 *62* 491-492
- FERNER H Das Inselssystem des Pankreas Thieme Verlag Stuttgart 1952
- FOLLIS R H JR Deficiency disease Charles C Thomas Springfield Illinois 1958
- FOSTER G V I MACVITYRE and A G E PEARSE Calcitonin production and the mitochondrion rich cells of the dog thyroid *Nature* 1964 *203* 1029-1030
- FOX F W and L F LEVY Experiments confirming the antiscorbutic activity of dehydroascorbic acid and a study of its storage and that of ascorbic acid by the guinea pig at different levels of intake *Biochem J* 1936 *30* 211-217
- FREEDLAND R A I M WADZINSKY and H A WARMAN The enzymatic hydroxylation of tryptophan *Biochem biophys Res Commun* 1961 *5* 94-98
- FRIBERG U Uptake of radiolabelled sulphate and radiophosphate in various tissues of normal and vitamin C-deficient guinea pigs *Ark kemi* 1958 *1* 481-500
- FRIEDENWALD J S W BUNCHAE and H O MICHEL Role of ascorbic acid (vitamin C) in secretion of intraocular fluid *Archs Ophthal* 1943 *29* 533-574
- FRIEDMAN G J S SHERRY and E P RALLY The mechanism of the excretion of vitamin C by the human kidney at low and normal plasma levels of ascorbic acid *J clin Invest* 1940 *19* 685-689
- FUJIMOTO D and N TAMURA Incorporation of O 18 from air into hydroxyproline by chick embryo *Biochem J* 1962 *84* 333-335
- FUJISAWA T M YAMAZAKI and H SAWAI Paper chromatographic microanalysis of vitamin C in foods *J Vitam* 1963 *9* 236-242
- FULLMER H M G R MARTIN and J J BURNS Role of ascorbic acid in the formation and maintenance of dental structures *Ann N Y Acad Sci* 1961 *92* 286-294
- GAL E M M POZIK and F D MARSHALL JR Hydroxylation of tryptophan to 5 hydroxytryptophan by brain tissue in vivo *Biochem biophys Res Commun* 1963 *12* 39-43
- GERSHON M ■ A B DRAGONIDIS and L L ROSS Serotonin Synthesis and release from the myenteric plexus of the mouse intestine *Science* 1965 *149* 197-199
- GIROUD A and C P FERNOND Etude histochemique de la vitamine C dans la glande surrénale *Archs Anat micros* 1934 *3* 106-129
- GIROUD A and A-R RATNANANCA Acide ascorbique Vitamine C Actual scient ind 1912 *921* 1-212

- GLIMCHER M J U A FRIBERG and P T LEVINE The identification and characterization of a calcified layer of coronal cementum in erupted bovine teeth J ultrastruct Res 1964 10 76-88
- GLIMCHER M J G MECHANIC L C BONAR and E J DANIEL The amino acid composition of the organic matrix of decalcified fetal bovine dental enamel J biol Chem 1961 236 3210-3213
- GLOCK G E H MELLANBY M MELLANBY M M MURRAY and J THEWLIS A study of the development of dental enamel in dogs J dent Res 194 21 183-199
- GLOOR F A PLETSCHER and TH HARDMEIER Metastasierendes Inselzelladenom des Pankreas mit 5-Hydroxytryptamin und Insulinproduktion Schweiz med Wschr 1964 94 1476-1480
- GOLDSMITH G A Human requirements for vitamin C and its use in clinical medicine Ann N Y Acad Sci 1961 92 230-245
- GOULD B S Ascorbic acid independent and ascorbic acid-dependent collagen forming mechanisms Ann N Y Acad Sci 1961 92 168-174
- GOULD B S and J F WOESSNER Biosynthesis of collagen The influence of ascorbic acid on the proline hydroxyproline glycine and collagen content of regenerating guinea pig skin J biol Chem 1957 226 289-300
- GRAB W Pharmakodynamische Wirkungen der Ascorbinsäure In »Ascorbinsäure« Ed K Lang Wiss Veroff dt Ges Ernähr 1965 14 136-148
- GRAHAM SMITH D G Tryptophan hydroxylation in brain Biochem biophys Res Commun 1964 16 586-592
- GREEN H and J L SAWYER Demonstration characterization and assay procedure of tryptophan hydroxylase in rat brain Analyt Biochem 1966 15 53-64
- GRULIN R C and H C SLAVIN Amino acid utilization in the synthesis of enamel and dentin matrices as visualized by autoradiography In »The use of radioautography in investigating protein synthesis« Ed C P Leblond and K B Warren Academic Press New York-London 1965 199-214
- HAMBERGER A and B HAMBERGER Uptake of catecholamines and penetration of trypan blue after blood brain barrier lesions Z Zellforsch 1966 70 386-392
- HAMMARSTRÖM L Autoradiographic studies of l-ascorbic and dehydro-l-ascorbic acid Abstr XIII Internat Congress of Physiological Sciences Tokyo 1965
- HAMMARSTRÖM L L E APPELREN and S ULLBERG Improved method for light microscopy autoradiography with isotopes in water soluble form Expl Cell Res 1965 37 608-613
- HAMMARSTRÖM L M RITZÉN and S ULLBERG Combined autoradiography and fluorescence microscopy Localization of labelled 5-hydroxytryptophan in relation to endogenous 5-hydroxytryptamine in the gastrointestinal tract Experientia 1966 22 213-215
- HAMMARSTRÖM L and S ULLBERG Specific uptake of C-alloxan in the pancreatic islets Nature In press 1966
- HARGI G K G A WILLIAMS A TENENHOUSE and C D ARNAUD Thyrocalcitonin Cytological localization by immunofluorescence Science 1966 15 73-75
- HARNDT R Die Verteilung der Ascorbinsäure in den Zahnkeimen menschlicher Embryonen Dt zahnärztl Z 1966 1 196-201
- HEIMANN W and A HEIMANN Mechanismus der Wirkung und der Enzymatischen Zerstörung der Ascorbinsäure. In »Ascorbinsäure« Ed K Lang Wiss Veroff dt Ges Ernähr 1965 14 211-228
- HILLMAN L and J J BURNS Metabolism of l-ascorbic acid (C<sup>14</sup>) in man J biol Chem 1958 230 923-930
- HEMPFL K Ausdehnung des Brenzkatechinaminspeichernden Zellsystems Verh dt Ges Path 1963 47 286-290
- HESS W C C Y LEE and B A NEIDIG The amino acid composition of enamel protein J dent Res 1953 32 585-587

- HOLLANDS B C S and S VANOV Localization of catechol amines in visceral organs and ganglia of the rat guinea pig and rabbit Br J Pharmac Chemother 1965 23 307-316
- HUGHES R E Reduction of dehydroascorbic acid by animal tissues Nature 1964 203 1068-1069
- HÄGGENDAHL J and T MALMFORS Evidence of dopamine containing neurons in the retina of the rabbit Acta physiol scand 1963 59 295-296
- — Identification and cellular localization of the catecholamines in the retina and the choroid of the rabbit Acta physiol scand 1965 64 58-66
- HOJER A Method for determining the antiscorbutic value of a foodstuff by means of histological examination of the teeth of young guinea pigs Br J exp Path 1926 7 356-360
- HOJER A and G WESTIN Jaws and teeth in scorbutic guinea pigs Dental Cosmos 1925 67 1-24
- HÖKFELT B Changes in the ascorbic acid content of the corpus luteum of pregnancy in the rabbit after gonadotrophic stimulation Acta physiol scand 1950 11 172-179
- JOHNSON S W and E S SILVA The urinary excretion of ascorbic and dehydroascorbic acids in man Biochem J 1934 28 1393-1408
- KAUFMAN S Coenzymes and hydroxylases Ascorbate and dopamine  $\beta$  hydroxylase tetrahydropteridines and phenylalanine and tyrosine hydroxylases Pharmac Rev 1966 18 61-69
- KAUFMAN E and S FRIEDMAN Dopamine  $\beta$  hydroxylase Pharmac Rev 1965 17 71-100
- KIECKERUSCH W W GRIEM and K LANG Untersuchungen über die chronische Toxizität der Ascorbinsäure bei der Ratte Z Ernähr Wiss 1963 4 5-14
- KING L S The hematoencephalic barrier Arch Neurol Psychiat 1939 41 51-72
- KINSEY V E Dehydroascorbic acid ascorbic acid in the aqueous humor of rabbits Am J Ophthalm 1950 33 257-266
- KRAUT H Der Ascorbinsäurebedarf der Menschen In »Ascorbinsäure« Ed K Lang Wiss Veröff dt Ges Ernähr 1965 14 89-98
- KUETHER C A I R TELFORD and J H ROE The relation of the blood level of ascorbic acid to the tissue concentrations of this vitamin and to the histology of the incisor teeth in the guinea pig J Nutr 1944 28 347-358
- LAMDEN M P and G A CHRYSTOWSKI Urinary oxalate excretion by man following ascorbic acid ingestion Proc Soc exp Biol Med 1954 85 190-192
- LANG K Wirkungen sehr hoher Ascorbinsäure Dosen In »Ascorbinsäure« Ed K Lang Wiss Veröff dt Ges Ernähr 1965 14 149-154
- LARSON H CH OWMAN and F SUNDLER Monoaminergic mechanisms in parafollicular cells of mouse thyroid gland Endocrinology 1966 78 1109-1114
- — Monoaminergic mechanisms in a special type of cells in the tracheal epithelium Z Zellforsch In press 1966
- LEFLOUND C B Recherches histochimiques sur la localisation et le cycle de la vitamine C dans l'organisme Laval 1934
- LEE R C Ascorbic acid and the peripheral vascular system Ann N Y Acad Sci 1961 9 295-301
- LEICESTER H M Biochemistry of the teeth C V Mosby Co St Louis 1949
- LEVIN F A B LEVENSBERG and S KAUFMAN The enzymatic conversion of 3,4-dihydroxyphenylethylamine to norepinephrine J Biol Chem 1960 235 2080-2086
- LLOYD B R and H M SINCLAIR Vitamin C In »Biochemistry and physiology of nutrition« Ed G H Bourne and S W Kaddet New York 1953 1 369-471
- LYNS L and C FROSTER Über den Einfluss der Dehydroascorbinsäure auf die bedingt reflektorische Tätigkeit bei Meerschweinchen Acta biol med germ 1963 11 182-188
- MACLEAN D I M SHEPPARD and F W McHENRY Tissue changes in ascorbic acid deficient guinea pigs Br J exp Path 1939 10 451-457
- MALMFORS T Evidence of a ferruginous iron with synaptic terminals in the retina of rats demonstrated by the use of electron microscopy Acta physiol scand 1963 58 99-100

- MANNERING G J Vitamin requirements of the guinea pig *Vitamins and Hormones* 1949 7 201--221
- MARTIN G R Studies on the tissue distribution of ascorbic acid *Ann N Y Acad Sci* 1961 92 141-147
- MARTIN G R and C E MECCA Studies on the distribution of l ascorbic acid in the rat *Archs Biochem Biophys* 1961 93 110-114
- MARTINI E A BONSIGNORE and F PINOTTI Acido ascorbico e deidroascorbico nello scorbuto sperimentale L'indice scorbutico *Quad Nutr* 1935 2 333-355
- MASUOKA D T H F SROTTE and L PETRIELLO Formation of catecholamines by various areas of cat brain *J Pharmac exp Ther* 1963 139 73-76
- MERLING D and F CARAMIA Effect of dehydroascorbic acid on the islets of Langerhans of the rat pancreas *J Cell Biol* 1965 16 245-261
- MOLL T and H VIETERS Untersuchungen über Dehydroascorbinsäure *M Mercks J ber* 1936 50 65-87
- MUSLIV R R and C G KING Metaphosphoric acid in the extraction and titration of vitamin C *J biol Chem* 1936 116 409-413
- NAUJOKS R Histotopochemische Untersuchungen am Zahnorgan *Dt Zahn Mund Kieferheilk* 1956 4 65-312
- NEUBRGER A and H G B SLACK The metabolism of collagen from liver bone skin and tendon in the normal rat *Biochem J* 1953 53 47-52
- NORBERG K A Adrenergic innervation of the intestinal wall studied by fluorescence microscopy *Int J Neuropharmac* 1964 3 379-382
- NORBERG K A and B HÄMBERGER The sympathetic adrenergic neuron *Acta physiol scand* 1964 63 suppl 238
- NORBERG K A and L OLSON Adrenergic innervation of the salivary glands of the rat *Z Zellforsch* 1965 68 183-189
- PATTERSON J W The diabetogenic effect of dehydroascorbic acid *Endocrinology* 1949 45 344
- The diabetogenic effect of dehydroascorbic and dehydroisoascorbic acids *J biol Chem* 1950 183 81-88
- PATTERSON J W and A LAZAROW Sulfhydryl protection against dehydroascorbic acid diabetes *J biol Chem* 1950 186 141-144
- PATTERSON J W and D W MARTIN Some effects of dehydroascorbic acid on the central nervous system *Am J Physiol* 1951 167 119 126
- PEARSE A G E The demonstration of ascorbic acid In »Histochemistry Theoretical and Applied« 2nd edit on J & A Churchill Ltd London 1961 271-280
- PENNEY J R and S S ZILVA The chemical behaviour of dehydro l ascorbic acid in vitro and in vivo *Biochem J* 1943 37 403-417
- The isolation of barium and calcium diketo l gulonates and the biological significance of a 3 diketo-l gulonic acid *Biochem J* 1945 39 1-4
- PFLEGER W Silberreduzierende Zellgranula und Gewebstrukturen und ihre Beziehungen zum Vitamin C-Stoffwechsel *Z mikrosk anat Forsch* 1941 50 299-338
- PIER K A Amino acid composition of some calcified proteins *Science* 1961 134 841-842
- PIRANT C L Relation of vitamin C to adrenocortical function and stress phenomena *Metabolism* 1952 1 197-222
- PLAUT F and M BULOW Vergleichende Untersuchungen über den C Vitamin (Ascorbinsäure) Gehalt im Blut und im Liquor cerebrospinalis *Hoppe Seylers Z physiol Chem* 1935 236 241-256
- PROCIJAZKA Z Ascorbinsäure und verwandte Substanzen In »Handbuch der Papierchromatographie« Ed I M Haus and K Macek Fischer Verlag Jena 1958 1 646-653



- PROCKOP D A KAPLAN and ■ UDENFRIEND Oxygen 18 studies on the conversion of proline to hydroxyproline *Biochem biophys Res Commun* 1962 9 16—166
- RITZEL G Klinische Beobachtungen von Ascorbinsäure Mangelzuständen und therapeutische Verwendung der Ascorbinsäure In »Ascorbinsäure« Ed K Lang Wiss Veroff dt Ges Ernähr 1965 14 125—135
- RITZÉN M L HAMMARSTROM and ■ ULLBERG Autoradiographic distribution of 5 hydroxytryptamine and 5 hydroxytryptophan in the mouse *Biochem Pharmac* 1965 14 313—321
- ROBERTSON WM VAN B The biochemical role of ascorbic acid in connective tissue *Ann N Y Acad Sci* 1961 92 159—167
- — Metabolism of collagen in mammalian tissues *Biophys J* 1964 4 suppl I 93—106
- ROE J H and G L BARNUM The anti scorbutic potency of reversibly oxidized ascorbic acid and the observation of an enzyme in blood which reduces the reversibly oxidized vitamin *J Nutr* 1936 11 359—369
- ROE J H and C A KUETHER The determination of ascorbic acid in whole blood and urine through the 2,4 dinitrophenylhydrazine derivative of d hydroascorbic acid *J Biol Chem* 1943 147 399—407
- ROSELL S G SEDVALL and ■ ULLBERG Distribution and fate of dihydroxyphenylalanine 2-<sup>14</sup>C (Dopa) in mice *Biochem Pharmac* 1963 12 65—269
- RUDOLFF S L R R BECKER and C G KING Synthesis and metabolism of l ascorbic acid 2,3,4,5,6-<sup>14</sup>C *Fed Proc* 1956 15 343
- SALOMON L I Studies on the metabolism under stress and the biosynthesis of ascorbic acid Dissertation Columbia University New York 1952
- — Catabolism of l ascorbic acid in guinea pigs *J Nutr* 1962 76 493—502
- SAYERS ■ M A SAYERS H L LEWIS and C N H LONG Effect of adrenotropic hormone on ascorbic acid and cholesterol content of the adrenal *Proc Soc exp Biol Med* 1944 51 238—239
- VON SCHLICHTING S L and A F ABT Verification of l ascorbic acid 1-<sup>14</sup>C catabolism to carbon<sup>14</sup> dioxide in the human by liquid scintillation counting *Proc Soc exp Biol Med* 1965 118 30—33
- SCHULTZE M O E STOTZ and C G KING Studies on the reduction of dehydroascorbic acid by guinea pig tissues *J Biol Chem* 1938 122 395—406
- SILAW J H Vitamin C deficiency in the ringtail monkey *Fed Proc* 1949 8 396
- SILAW J H P H PHILLIPS and C A ELVEIJEM Acute and chronic ascorbic acid deficiencies in the rhesus monkey *J Nutr* 1945 9 365—372
- SIGAL A and C G KING The relationship of vitamin C to glucose tolerance in the guinea pig *J Biol Chem* 1936 116 489—492
- SLUSHER M A and ■ ROBERTS Fate of adrenal ascorbic acid Relationship to corticosteroid secretion *Endocrinology* 1957 61 98—105
- SNYDER S ■ J AXELROD and M ZWISLOCKY A sensitive and specific fluorescence assay for tissue serotonin *Biochim Pharmac* 1965 14 831—833
- SNYDER F and A STEPHENS Quantitative carbon 14 and tritium assay of thin layer chromatography plates *Analyt Biochem* 1962 4 128—131
- STACK M V Organic constituents of enamel *J Am dent Ass* 1954 48 97—306
- STÄHL E *Dünnschicht-chromatographie* Springer Verlag Berlin—Göttingen—Heidelberg 1962
- STEWART C P D ■ HORY and J ■ ROBSON Dehydroascorbic acid in human blood plasma *Proc Nutr Soc* 1953 12 304 305
- THORELL B and A WILTON The nucleotide metabolism of the dentine cells under normal conditions and in avitaminosis C *Acta path microbiol scand* 1945 22 593—602
- TODHUNTER E A T McMILLAN and D A FIMKE Utilization of dehydroascorbic acid by human subjects *J Nutr* 1954 7 97 108
- TOVERLID ■ The influence of l-ascorbic acid on bone *J Biol Chem* 1923 58 583—600

- UDENFRIEND S Formation of hydroxyproline collagen Science 1966 152 1335-1340
- UDENFRIEND S and C R CREVELING Localization of dopamine  $\beta$  oxidase in brain J Neurochem 1959 4 350-352
- ULLBERG S Studies on the distribution and fate of S <sup>35</sup> labelled benzylpenicillin in the body Acta Radiol 1954 suppl 118
- — Autoradiographic studies on the distribution of labelled drugs in the body Second U N Int Conf Peaceful Uses of Atomic Energy 1958 24 248-254
- VOGT M Ascorbic acid in adrenal blood J Physiol (London) 1948 107 239-243
- — The concentration of sympathin in different parts of the central nervous system under normal conditions and after the administration of drugs J Physiol (London) 1954 123 451-481
- WAGNER A Experimenteller Menschweinchenskorbut bei verschiedenen Kostarten mit besonderer Berücksichtigung der Zahnbefunde Dt Mischr Zahnheilk 1932 50 721-745
- WASSERMANN F Analysis of the enamel formation in the continuously growing teeth of normal and vitamin C deficient guinea pig J dent Res 1944 23 463-509
- WEGLANN A Zentrale und periphere Sympathicusaktivierung durch Dehydroascorbinsäure Acta physiol scand 1958 4 363-370
- WERNHART J P G D WESINGER and G REED Correlation of chemical and histological investigations on developing enamel J dent Res 1942 21 171-182
- WISLOCKI G B and E H LEDUC Vital staining of the hematoencephalic barrier by silver nitrate and trypan blue and cytological comparisons of the neurohypophysis pineal body area postrema intercolumnar tubercle and supraoptic crest J comp Neurol 1952 96 371-413

## CONTENTS

General introduction	3
Distribution of ascorbic acid 1 C <sup>14</sup> in adult mice	5
Materials and methods	5
Results	8
Distribution of dehydroascorbic acid 1 C <sup>14</sup> in adult mice	21
Materials and methods	23
Results	24
Distribution of ascorbic acid 1 C <sup>14</sup> and dehydroascorbic acid 1 C <sup>14</sup> in suckling rats	33
Materials and methods	33
Results	34
Chromatographic investigation of selected tissues after injection of ascorbic acid 1 C <sup>14</sup> and dehydroascorbic acid 1 C <sup>14</sup>	47
Materials and methods	48
Results	50
Comparison of the distribution patterns of ascorbic acid 1 C <sup>14</sup> and dehydroascorbic acid 1 C <sup>14</sup> as determined by impulse counting	53
Materials and methods	53
Results and comments	54
Discussion	59
Summary	73
Acknowledgements	75
References	76





ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 70 SUPPLEMENTUM 290

STUDIES ON THE PREAORTAL  
PARAGANGLIA OF NEWBORN  
RABBITS

BY

TÓMAS BRUNDIN

STOCKHOLM 1966



ACTA PHYSIOLOGICA SCANDINAVICA

*Vol 70 Supplementum 290*

FROM THE DEPARTMENT OF PHYSIOLOGY  
KAROLINSKA INSTITUTET  
STOCKHOLM SWEDEN

STUDIES ON THE PREAORTAL  
PARAGANGLIA OF  
NEWBORN RABBITS

BY

TOMAS BRUNDIN

STOCKHOLM 1966





# CONTENTS

INTRODUCTION	5
CHAPTER I HISTORICAL SURVEY	7
CHAPTER II GENERAL METHODS	11
Material	11
Technique of dissection	11
Organ weights	11
Extraction of catecholamines	12
Fluorimetric determinations of noradrenaline and adrenaline	13
Separation of catecholamines	13
Radioactivity measurement	14
CHAPTER III STUDIES ON THE CATECHOLAMINE STORES IN THE PARAGANGLIA	15
Special methods	15
Identification of catecholamines in paraganglia	15
Histochemical studies	15
Differential centrifugation of homogenates of paraganglia and adrenal glands	16
Electron microscopical studies	16
Results	17
Identification of catecholamines in paraganglia	17
Noradrenaline amounts in paraganglia from rabbits of dif ferent perinatal ages	19
Histochemical observations	21
Distribution of catecholamines in sediments and superna tants after high speed centrifugation of homogenates of pa ranganglia and adrenal glands	21
Electron microscopical observations	22
Discussion and conclusions	22

CHAPTER IV	STUDIES ON THE FORMATION OF CATECHOLAMINES IN THE PARA GANGLIA	30
	Special methods	30
	Incorporation of radioactive catecholamines from C <sup>14</sup> label ed precursors	30
	Uptake of H <sup>3</sup> labeled noradrenaline	31
	Results	31
	Incorporation of C <sup>14</sup> labeled catecholamines after pretreat ment with dihydroxyphenylalanine C <sup>14</sup>	31
	Incorporation of C <sup>14</sup> labeled catecholamines after pretreat ment with tyrosine C <sup>14</sup>	35
	Uptake of noradrenaline	35
	Discussion and conclusions	39
CHAPTER V	EFFECTS OF CELLULAR GLUCOPENIA AND ASPHYXIA UPON THE CATE CHOLAMINE CONTENTS IN PARAGANGLIA AND ADRENAL GLANDS	41
	Special methods	41
	Cellular glucopenia	41
	Asphyxia	42
	Hypoxia	42
	Hypercapnia	42
	Statistical methods	42
	Results	43
	Effects of cellular glucopenia upon the catecholamines in paraganglia and adrenal glands	43
	Effects of asphyxia upon the catecholamines in paraganglia and adrenal glands	44
	Discussion and conclusions	45
	SUMMARY	48
	ACKNOWLEDGEMENTS	50
	REFERENCES	51

## INTRODUCTION

The preaortal paraganglia (or Zuckerlandl organs ) constitute large sources of catecholamines in fetal and newborn mammals. The physiological role of these organs has however not yet been clarified — In the present work the preaortal paraganglia of newborn rabbits have been investigated with regard to their storage, synthesis and release of catecholamines. Most experimental series have been performed as comparative studies using the adrenal glands of the same animals as reference organs.



## CHAPTER I

### HISTORICAL SURVEY

Chromaffin tissue containing catecholamines (adrenaline noradrenaline and in some species dopamine) has been found in all mammalian species studied (Euler 1956). Owing to the important biological effects of the catecholamines for maintaining a physiological homeostasis especially in stressful situations the localization and functions of the chromaffin tissues have been extensively studied (for ref. see West 1955, Coupland 1965). Chromaffin cells have thus been found partly as small groups of cells scattered in various organs throughout the body and partly in the form of a highly specialized organ, the adrenal medulla. In adult animals this organ constitutes the main source of catecholamines.

In fetal and newborn mammals however an additional type of chromaffin tissue has been identified in the vicinity of the adrenal glands. Dostoevsky (1886) originally demonstrated the presence of this extra-adrenal chromaffin tissue in several mammalian species describing it as *accessorische primitive Nebennieren*. It was not until 1901 however that Zuckerkandl was able to demonstrate that this tissue in fetal and newborn humans, besides occurring as small islets of chromaffin cells around the adrenal glands, also constitutes a well-defined paired retroperitoneal organ on the ventral wall of the abdominal aorta. In subsequent literature these organs have frequently been referred to as Zuckerkandl organs. Kohn (1903) performed the first detailed comparative study on the anatomical relationships between the adrenal medulla and the paraganglion aorticum abdominale or paraganglia, as he named the extra-adrenal chromaffin tissue. Kohn showed that the embryonic development and final anatomical arrangement of the paraganglia are different in different mammalian species. Thus in man and cat the paraganglia are formed by ectodermal cells migrating forward from the neural crest to both sides of the aorta where they form large clusters of chromaffin cells surrounded by smaller islets of similar cells. In these species there is no direct anatomical continuity between the chromaffin cells of the paraganglia and those of the adrenal medulla. In dogs an almost identical development

was described by Kahn (1912), whereas in rabbits as shown by Kohn (1903) both the paraganglionic and adrenomedullary cells develop within one and the same morphological unit. Thus the abdominal chromaffin cells in the rabbit embryo form two strands of cells one on each side of the abdominal aorta. At about 15 days of gestation the cranial portions of these strands become partly enclosed by the adrenocortical germ cells. The caudal portions melt together onto the ventral wall of the aorta into one slightly bipartite mass of chromaffin cells which then constitutes the paraganglion. The direct anatomical connection between the paraganglion and the adrenal medulla of both sides persists even some time after birth. — As compared to those other mammalian species studied the paraganglionic cells of the rabbit are considerably more confined to the large preaortal cell masses the surrounding groups of accessory chromaffin cells being few and small.

In humans the paraganglia undergo fibrotic degeneration soon after birth, and at the age of 3 to 5 years connective tissue occupies almost the entire organ (Zuckerhandl 1901, 1911). However the information on the postnatal fate of the paraganglia in other mammals is to some extent controversial. According to Kohn (1903) the paraganglia of cats and rabbits persist throughout life (*cf.* Coupland 1956). On the other hand Pellegrini (1906) found degenerative changes in the paraganglionic tissue of several mammals: dog and mouse as well as cat and rabbit. In rats an almost total degeneration of the paraganglia occurs soon after birth (Coupland 1960 and others). Recently Lempiäinen (1964) reported that this postnatal involution of the rat's paraganglia could be prevented or inhibited by the administration of glucocorticoid hormones.

On the functional role of the paraganglia our information is very limited. Biedl and Wiesel (1902) showed that extracts of paraganglia from newborn humans contained substances with pressor effects similar to those previously obtained with adrenal extracts (Oliver and Schafer 1895). Paraganglia from cats and dogs were also found to contain pressor substances (Vincent 1910, Kahn 1912 and others). Lelkes (1911) using a bioassay technique observed that in the same human *fetus* the amounts of pressor substances in the paraganglia exceeded those found in the adrenal glands. Similar quantitative results have been reported by several authors (for ref. see West *et al.* 1953). Separate determinations of adrenaline and noradrenaline revealed that the pressor substances almost exclusively consisted of noradrenaline in the paraganglia of all the mammalian species studied: man, dog, cat, rabbit and guinea pig (Shepherd and West 1952, Nieminen and Pekkarinen 1952 and others). Traces of adrenaline were also found in the paraganglia of certain

specimens. No precursors or metabolites of catecholamines were demonstrated in the paraganglia.

In the adrenal glands of fetal and newborn animals the ratio of noradrenaline/adrenaline was found to be higher than in the adult (West and Hunter 1951, Hofk 1951 and others). Shepherd and West (1951) reported that in the adrenal glands of fetal rabbits no adrenaline could be recovered and that the total catecholamine content consisted of noradrenaline. However, recently two independent studies on the adrenal catecholamines of the developing rabbit *fetus* showed that after 24 days of gestation the adrenaline content far exceeded that of noradrenaline (Roffi 1964, Brundin 1965 a).

The subcellular organization of the catecholamine storing organs has become the object for extensive studies in recent years. It is now well established that in the adrenomedullary cells as well as in sympathetic nerve endings the catecholamines are bound to cytoplasmic osmophilic granules visible in the electron microscope (Hillarp, Lagerstedt and Nilson 1953, Blaschko and Welch 1953, Lever 1955, Euler and Hillarp 1956, Lever and Esterhuizen 1961). It has also been described that the granules in adrenomedullary cells differ from nerve granules with respect to both their catecholamine binding properties and their reactions to certain pharmacological agents (for ref. see Stjärne 1964). Concerning the subcellular organization of the paraganglionic tissue, however, no information seems to be available either with regard to its mechanisms for catecholamine storage or to its ultrastructure in general.

Since Gurin and Delluva (1947) were able to isolate  $C^{14}$  labeled adrenaline from the adrenal glands of rats pretreated with phenylalanine  $C^{14}$ , the biosynthesis of catecholamines in the adrenal medulla has been intensely studied. In experiments using both *in vivo* and *in vitro* techniques the pathway of catecholamine formation from tyrosine via dihydroxyphenylalanine and dopamine previously outlined by Blaschko (1939) has been confirmed by several investigators (for ref. see Kirshner 1958, Udenfriend 1965). After studies on the different steps of the synthetic pathway the conversion of tyrosine to dihydroxyphenylalanine was found to be a time consuming process, whereas the subsequent steps: decarboxylation to dopamine, hydroxylation to noradrenaline and methylation to adrenaline have been regarded as rapid reactions (Rosenfeld, Leeper and Udenfriend 1958). — There is no information available regarding the capacity of noradrenaline synthesis in the paraganglia. Nor is it known if the paraganglia possess any mechanisms for selective uptake of noradrenaline. In the adrenal medulla the uptake of injected adrenaline has been found to be low (Udenfriend and Wyngaarden 1956).

It is well known that the release of catecholamines from the adrenal medulla into the blood stream can be elicited by stimulation of the splanchnic nerves.



(Dreyer 1899) Thus the dramatic increase of adrenaline release during hypoglycemic conditions seems to be mediated exclusively by this innervation (for ref. see Duner 1953) On the other hand certain pathophysiological conditions such as asphyxia and hypoxia have been found to cause increased release of catecholamines also from denervated adrenal glands (Comline and Silver 1958, 1961, Comline Silver and Silver 1963) Recently this non nervous or direct stimulation of the adrenal medulla has been found more easily demonstrable in fetal and newborn animals than in the adult (Comline and Silver 1966) — The question whether or not the paraganglionic tissue is furnished with any functional innervation has not yet been definitely clarified In morphological studies on dogs and cats Hollinshead (1937) has described nerve fibres with terminals in the paraganglionic tissue Since no such nerve fibers could be observed after section of the *rami communicantes* of the lumbar segments it was assumed that the paraganglia had a preganglionic innervation similar to that of the adrenal medulla However by means of electrical stimulation of the inferior splanchnic nerves of young dogs no support was obtained for any functional preganglionic innervation of the paraganglionic tissue (Muscholl and Vogt 1964)

The paraganglia are supplied with a dense vascular net (Kolin 1903 and others) Therefore substances released from the paraganglionic cells into the blood could easily be distributed in the organism However it is not yet known whether or not the paraganglia are able to release catecholamines under physiological or pathophysiological conditions

---

The following abbreviations have been used

A	= adrenaline
CA	= catecholamine(s)
DA	= dopamine
DOPA	= dihydroxyphenylalanine
NA	= noradrenaline
f u	= fluorescence units

## CHAPTER II

### GENERAL METHODS

#### Material

Albino rabbits were used. For good access of fetal and newborn animals a rabbit breeding was organized. The rabbits were mated under observation. The time of gestation was regularly 30—31 days (*cf.* Asdell 1946). The deliveries observed seldom exceeded 4 hrs. Most litters contained 6—8 youngs of 35—55 g each. When the newborn rabbits were kept apart from their mothers in the nest at room temperature, their body temperature (intracolic) was relatively constant, 34.0—36.5°C. In those experiments where an experimental group of animals has been compared to a control group both groups have been recruited from the same litter.

All the experiments were performed from March to October. During the winter no experiments were carried out because only few youngs were obtained from the breeding at this time of the year when many doe rabbits where in anestrus. Pseudopregnancies also occurred frequently during the winter.

#### Technique of dissection

In the studies on *foetus* the doe rabbit was killed by a blow on the head and the *foetus* were immediately removed and decapitated. The newborn animals were killed by decapitation. Through a large abdominal incision the preaortal paraganglion was carefully dissected from the adjacent tissues under a dissection microscope. The paraganglion was separated from the adrenal glands as shown in Fig. 1. The thin cranial section of the paraganglion extending to the right adrenal gland was left in the animal since it was covered by liver tissue and impossible to excise accurately. In those experiments where the adrenal glands were also studied these were dissected after the removal of the paraganglia.

#### Organ weights

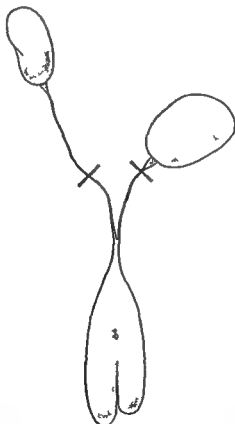
The paraganglia and adrenal glands from one litter of 7 newborn rabbits were weighed on a balance (Mettler type H 16) with a sensitivity of  $\pm 0.02$  mg.

During the short interval between dissection and weighing the organs were kept in an ice cold metal bowl in order to reduce weight loss caused by evaporation. The mean weight of the paraganglion was 2.9 mg with a range of 2.0—5.3 mg. The adrenal pairs (right and left) had a mean weight of 6.9 mg with a range of 2.7—9.6 mg.

Because of this wide variation in organ weights all CA values are expressed as  $\mu\text{g}$  per paraganglion or per pair of adrenal glands.

### Extraction of catecholamines

Immediately after the excision the paraganglion and adrenal glands were ground separately with chemically pure quartz sand in 2 ml of 0.1 M per



*Fig. 1. Paraganglion and adrenal glands from a newborn rabbit. Double lines indicate the levels where the paraganglion was cut and separated from the adrenal glands.*

In one case the paraganglion was found aberrant, being divided into cranial and caudal halves separated by a diastasis of about 2 mm.

chloric acid. Unless stated otherwise the paraganglia were extracted one by one, the adrenal glands by pairs. The extracts were stored at  $-20^{\circ}\text{C}$  until analysis. When stored more than one week or when the CA of the extracts were to be separated chromatographically, the extracts were adjusted to pH 4 (cf. Gunne 1963) by titration with 2 N potassium hydroxide and centrifuged at  $15,000 \times g$  and  $0^{\circ}\text{C}$  for 10 min to remove precipitating potassium perchlorate. The extracts were thoroughly stirred during thawing in order to avoid separation caused by cryoscopic effects (Rangappa 1961).

### Fluorimetric determinations of noradrenaline and adrenaline

The concentrations of NA and A in the extracts were determined photo-fluorimetrically using the trihydroxyindole method (Ehrlén 1948, Lund 1949, Euler and Lishajko 1959) in which the CA are oxidized and rearranged to their respective lutines, the fluorescence of which is measured.

Due to the high concentrations of CA in the actual extracts they could be assayed directly without any preceding purification procedure. After centrifugation at  $800 \times g$  at room temperature for 5 min, 0.5 ml of the extracts was adjusted to pH 6.5 by addition of 2.5 ml 0.25 M sodium phosphate buffer and oxidized with 0.1 ml 0.25% potassium ferricyanide solution for 3 min. The oxidation was interrupted with 4 ml 0.2% ascorbic acid in 5 N sodium hydroxide. Ethylene diamine was added in order to stabilize the fluorescence according to Euler and Lishajko (1961). The fluorescence was measured in a photofluorometer (Turner model 110) with 2 sets of filter combinations (Schott 390 nm/Ilford Bright 490 nm and Schott 436 nm/Corning 540 nm). The mean recovery for added NA and A was 97% with a range of 94–101% in several control tests. The NA and A values are expressed as the bases of the respective amines.

### Separation of catecholamines

Separation of NA, A and DA was performed by means of an ion exchange chromatographic technique according to Haggendal (1962). A strong cation exchange resin (Amberlite CG 120 type 2 mesh 200–400) was used in Na form. Separation of NA, A and DA was performed on resin columns of  $15 \times 0.4$  cm. When only NA and DA were to be separated  $5 \times 0.4$  cm columns were used. The organ extracts were of about 3 ml and adjusted to pH 4. After adsorption of the extract the column was washed with 3 ml 1 M sodium acetate buffer pH 4 and 3 ml redistilled water. A pressure of 100 mm Hg was applied during the adsorption and rinsing processes but not during the

subsequent elution. The elution of NA and A from the 15 cm columns was performed with 0.4 N hydrochloric acid. DA was then eluted with 2 N hydrochloric acid from the same columns. From the 5 cm columns NA and DA were eluted with 1 N hydrochloric acid. To protect the amines from oxidation in the columns, ascorbic acid, 10  $\mu\text{g}/\text{ml}$  was added to the eluant. The eluate was separated into 1.1 ml fractions by a fraction collector with drop counter (LKB Radi Rac). The direct fluorescence of each fraction was measured at 285/335 nm wavelengths (activ/fluor instrumental values) in an Aminco Bowman spectrophotofluorometer and compared to a NA standard solution (1  $\mu\text{g}$  NA/ml = 50 f.u.). By this method the following recovery values were obtained:

15 cm columns, NA and A 70–80% DA 35–55 %

5 cm columns NA 80–90 % DA 35–45 %

### Radioactivity measurement

In certain experiments the radioactivity was measured in the fractions of the chromatograms. A liquid scintillation technique was employed. Sample volumes of 500  $\mu\text{l}$  were diluted with 6 ml absolute ethanol and 14 ml toluene containing 4 g/l of 2,5-diphenyloxazole and 0.10 g/l of 1,4-bis(2,5-phenyloxazolyl)benzene and counted for 10 min in a liquid scintillation spectrometer (Packard Tri Carb). Several series of samples were counted in duplicates and showed good agreement even with activities below 100 cpm. The radioactivity of each fraction in the chromatograms is expressed as cpm. The background radioactivity never exceeded 750 counts when repeatedly controlled by counting blank samples for 10 min on the C<sup>14</sup> channel. The instrumental background level was therefore set to 75 cpm.

## CHAPTER III

# STUDIES ON THE CATECHOLAMINE STORES IN THE PARAGANGLIA

### Special methods

#### Identification of catecholamine in paraganglia

In extracts of pooled paraganglia from newborn rabbits NA, A and DA were analyzed after separation on 15 cm Amberlite columns. After measurement of the direct fluorescence of each fraction the fractions at the positions of NA, A and DA were adjusted to pH 6.5 by the addition of 0.5 M potassium phosphate buffer. The NA and A fractions were then oxidized with potassium ferricyanide and the DA fractions were oxidized with iodine (*cf* Euler and Lishajko 1959, 1961; Carlsson and Waldeck 1958). The fluorescence of the oxidation products was read at 410/535 nm for NA, at 425/545 nm for A and at 345/410 nm for DA (uncorrected instrumental wavelength values) in an Aminco Bowman spectrophotofluorometer.

Corresponding chromatographic separations were performed on extracts of the paraganglion and the adrenal glands from a one day old rabbit which had received dl dihydroxyphenylalanine 2 Ci<sup>14</sup>, 0.12 µCi/g b.w. s.c. 12 hrs before the dissection. A and DA were added to the paraganglion extract before the separation. The CA were localized in the chromatograms by measuring the direct fluorescence of each fraction. The radioactivity of each fraction was measured by liquid scintillation.

#### Histochemical studies\*

In order to study the localization of CA in the paraganglia at different stages of postnatal development a histochemical study was undertaken. A total of

---

\* These studies were performed in collaboration with B. Hamberger and K. A. Norberg at the Department of Histology, Karolinska Institutet.

14 paraganglia from newborn and young rabbits were treated according to the formaldehyde condensation method of Falck and Hillarp (Falck 1962 Falck *et al* 1962). By this method CA are condensed with formaldehyde to intensely fluorescent products. The chemical basis for the reaction and its specificity and sensitivity when used in tissues has been described and discussed by Corrodi and Hillarp (1963, 1964). For technical procedures see Norberg and Hamberger (1964), Falck and Owman (1965).

### Differential centrifugation of homogenates of paraganglia and adrenal glands

Three experiments were performed on organs from 15 newborn rabbits. In each experiment 5 pooled paraganglia and 10 pooled adrenal glands were used. After the excision the organs were immediately immersed in 10 ml ice cold 0.13 M potassium phosphate buffer pH 7.4. The subsequent preparation was performed at +4°C. The pooled organs were then homogenized by grinding with celite (mesh 30–80). The homogenates were centrifuged at  $1\,000\times g$  for 10 min to remove coarse particles. From the approximate 10 ml of low speed supernatants obtained, 2 ml aliquots were centrifuged at  $50\,000\times g$  for 30 min in a refrigerated centrifuge (MSE Super Speed 25). The high speed supernatants were decanted in 0.5 ml 0.4 M perchloric acid and adjusted to pH 3.5 by titration with 2 M potassium hydroxide. Each high speed sediment was extracted with 1 ml 0.4 M perchloric acid. The CA contents of the extracts were determined fluorimetrically.

### Electron microscopical studies\*

Paraganglia and adrenal glands from 5 newborn rabbits were studied by electron microscopic technique. Under light ether anaesthesia fixation of the organs was instigated *in situ* by dripping the fixative onto the exposed organs for 5 min. The fixative used was a cold 1% solution of osmium tetroxide buffered with Veronal acetate to pH 7.2–7.4 and made approximately isotonic to mammalian plasma. After excision of the organs the fixation was continued *in vitro* for 3 hrs. Rinsing in buffer solution and slow dehydration in acetone preceded embedding in Vestopal W. The sections were examined in an RCA EML 3A electron microscope.

---

\*These studies were performed in collaboration with S. F. C. Nelson at the Department of Anatomy, Karolinska Institute.

## Results

### Identification of catecholamines in paraganglia

In extracts of 2, 2 and 3 paraganglia from 7 newborn rabbits the contents of NA, A and DA were measured after chromatographic separation. The chromatograms (Fig. 2) show that the direct fluorescence reached high values in the fractions at the position of NA. No other peaks were observed.

After oxidation, high fluorescence values at the maximum wavelengths for NA (410/535 nm) occurred in the same fractions which gave the direct fluorescence peaks. In the subsequent fractions, corresponding to the position of A, the fluorescence at the maximum wavelengths for A (425/545 nm) gave blank values. Neither was there any measurable fluorescence at the maximum wavelengths for DA (345/410 nm) in those fractions where this amine would be expected.

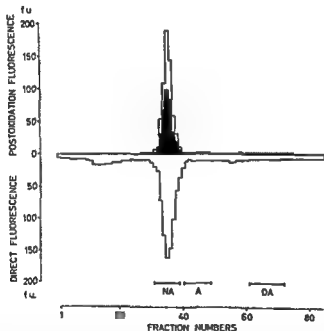


Fig. 2 Chromatographic distribution of direct fluorescence at 285/335 nm and postoxidation fluorescence at 410/535 nm (□) 425/545 nm (■) and 345/410 nm (▨) after separation of CA in an extract of 2 pooled paraganglia of newborn rabbits. Fraction collection started at the adsorption of the extract on the column. Horizontal bars indicate the expected positions for NA, A and DA. Column length: 15 cm.



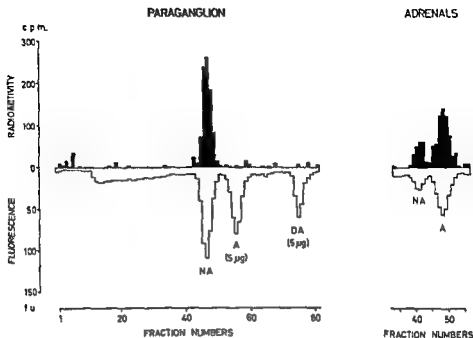


Fig 3 Chromatographic distribution of radioactivity and direct fluorescence after separation of CA in extracts of paraganglion and adrenal glands from a 1 day old rabbit 12 hrs after administration of DOPA C<sup>14</sup>. Adrenaline (A) and dopamine (DA) added to paraganglion extract. Fraction collections started at the adsorption of extract on the column. Column length 15 cm.

In order to detect even trace amounts of A and DA the radioactivity of each fraction was measured after an identical chromatographic separation performed on a paraganglion extract from a one day old rabbit pretreated with a radioactively labeled CA precursor, DOPA C<sup>14</sup>. Unlabeled A and DA were added to the extract before the separation.

A peak of radioactivity coincided with the direct fluorescence at the position of NA (Fig 3) while barely measurable traces of radioactivity were found in the A and DA fractions of the paraganglionic chromatogram. However in the chromatogram of the adrenal glands from the same animal, peaks of radioactivity were obtained both in the NA and A fractions. A C<sup>14</sup> being quantitatively dominant.

Table I CA contents in paraganglia ( $\mu\text{g}/\text{organ}$ ) and adrenal glands ( $\mu\text{g}/\text{pair}$  of organs) from one litter of 6 fetal rabbits at 29 days of gestation

Foetus nr	PARAGANGLIA	ADRENALS	
	NA	NA	A
1	4.72	0.71	2.18
2	6.13	0.34	2.35
3	4.69	0.28	1.10
4	4.03	0.53	1.81
5	2.21	0.49	1.55
6	5.22	0.73	1.71
Mean $\pm$ S.E.M.	$4.50 \pm 0.32$	$0.51 \pm 0.07$	$1.49 \pm 0.19$

# Noradrenaline amounts in paraganglia from rabbits of different perinatal ages

The NA contents were measured fluorimetrically in the paraganglia from a total of 6 fetal and 32 young rabbits, 1 day to 4 week old. In the fetal series the contents of CA in the adrenal glands were also measured for comparison. The results are listed in Tables I—III in terms of  $\mu\text{g}/\text{animal}$ .

The foetus were removed from the uterus 29 days after mating (day of mating not included). The mean CA content in the paraganglia was about twice that of both the adrenal glands from the same foetus (Table I). The NA values for the fetal organs showed small variations with one exception foetus nr 5.

Table II Contents of NA ( $\mu\text{g}$  per organ) in paraganglia from 5 litters (a—e) of 24 rabbits 1—7 days of age

	1 DAY		2 DAYS		5 DAYS		7 DAYS			
Litter	Animal nr	NA	Animal nr	NA	Litter	Animal nr	NA	Litter	Animal nr	NA
a	1	6.72	4	7.41	b	13	8.42	d	19	6.74
	2	6.38	5	6.77		14	6.98		20	10.0
	3	6.70	6	7.10		15	6.11		21	10.4
b	7	7.21	10	7.73	c	16	6.61	e	22	3.20
	8	5.94	11	6.98		17	7.78		23	5.60
	9	6.38	12	7.20		18	6.61		24	8.43
Me	$\pm$ S.E.M.	6.47 $\pm$ 0.18		7.0 $\pm$ 0.14		7.09 $\pm$ 0.35		7.42 $\pm$ 1.1		

Table III NA in paraganglia ( $\mu\text{g}$  per organ) from 2 litters of rabbits 2 and 4 weeks of age

2 WEEKS (litter f)		4 WEEKS (litter g)	
Animal nr	NA	Animal nr	NA
25	137	28	461
26	130	29	111
27	50	30	232
		31	500
		32	243
		Mean $\pm$ S.E.M.	$371 \pm 0.58$

The NA contents in the organs from 1 day old rabbits (Table II) far exceeded those in the fetal organs and in the 2 and 5 day old rabbits the values were further increased. In the paraganglia of 7 day old animals the mean content of NA was essentially unchanged, but the individual values showed considerably wide variations. In two of the 2 week old rabbits (Table III) the NA

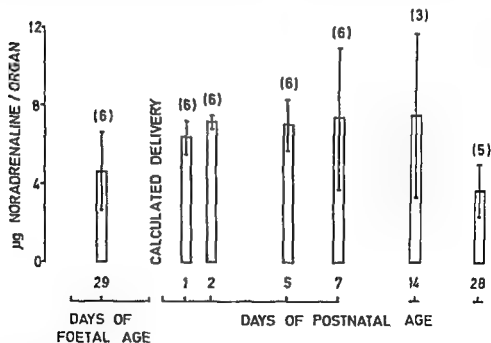


Fig. 4 Total contents of NA in paraganglia from rabbits at different stages of perinatal development. Mean and range values.

values were slightly reduced but in one specimen a remarkably high value was obtained. After 4 weeks there was a definite decrease in the NA content the mean value being even lower than in the fetal organs.

Fig. 4 gives a summary of the mean contents of NA in the paraganglia during the perinatal period studied.

### Histochemical observations\*

In order to find out whether the localization of CA in the paraganglia changed during the postnatal period of decreasing NA content in the organs a histochemical study was performed on paraganglia from newborn and young rabbits.

In newborn rabbits (Fig. 5) the paraganglia consisted of strands of polygonal or ovoid cells surrounded by a fibrous capsule. The organ was divided by the capsule into two parts (*cf.* Chapter I). The cytoplasm of the parenchymatous cells exhibited an intense yellow-green fluorescence corresponding to high concentrations of CA. This intense fluorescence was observed in all the cells throughout the parenchyma.

Essentially the same pictures were obtained from paraganglia sections of rabbits two weeks old but some clusters of cells with lower fluorescence intensity were also observed suggesting that these cells had partially lost their CA contents.

In rabbits two months old the paraganglia were no longer enclosed by proper capsules. Connective and to some extent, fatty tissue occupied almost the entire organ. Only small islets of intensely fluorescent cells remained (Fig. 6).

### Distribution of catecholamines in sediments and supernatants after high speed centrifugation of homogenates of paraganglia and adrenal glands

The subcellular distribution of CA in paraganglia and adrenal glands of newborn rabbits was studied by differential centrifugation of organ homogenates. The amounts of CA recovered from the sediments and supernatants obtained by high speed centrifugation are listed in Table IV. In the paraganglionic homogenates 68—76 % of the total CA contents were recovered from the high speed sediments. In the homogenates of the adrenal glands from the same animals 76—83 % of the total CA contents were bound to

---

\* A preliminary report of these observations has been published (Brundin, Hamberger and Norberg 1966).

*Table IV* CA ( $\mu$ g) in high speed sediments (sed) and supernatants (su) from homogenates of pooled paraganglia and adrenals of newborn rabbits

Exp nr	NA in paraganglia homogenates				NA + A in adrenal homogenates			
	sed	su	total	$\frac{\text{sed}}{\text{total}} \%$	sed	su	total	$\frac{\text{sed}}{\text{total}} \%$
I	4.78	1.76	6.54	73	2.74	0.76	3.50	78
II	5.88	2.77	8.65	68	3.03	0.96	3.99	76
III	6.74	2.13	8.87	76	4.01	0.81	4.82	83

the sedimentable fraction. Thus the sedimentable portions of CA in the adrenal homogenates slightly exceeded those of the paraganglionic homogenates. This difference was however not statistically significant.

### Electron microscopical observations\*

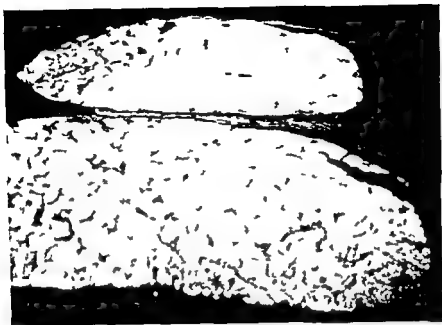
In cells of paraganglia as well as of adrenal medulla from newborn rabbits large amounts of membrane limited osmiophilic granules were found (Fig 7—9). The adrenomedullary granule consisted of a relatively large electron dense core and a less electron dense dotted periphery. The paraganglionic granule exhibited a considerably smaller electron dense core and a larger peripheral zone of low opacity. These differences in appearance between the granules from the two organs were consistently observed in the sections from all the animals studied. However the granules of both organs were of about the same size, their average diameters being about 0.15  $\mu$ .

### Discussion and conclusions

The CA stores in paraganglia from newborn rabbits were found to consist of NA exclusively. This amine was identified in extracts of pooled organs by different methods, all giving congruent results. No A or DA was recovered from the extracts.

In earlier investigations of the CA content in paraganglionic tissues from several mammalian species, small amounts of A have been found in some specimens (Shepherd and West 1952; Nummenya and Pekkarinen 1952; Greenberg and Lind 1961 and others). Also in two preliminary reports (Brundin

\* A preliminary report of these observations has been published (Brundin and Nilsson 1965).

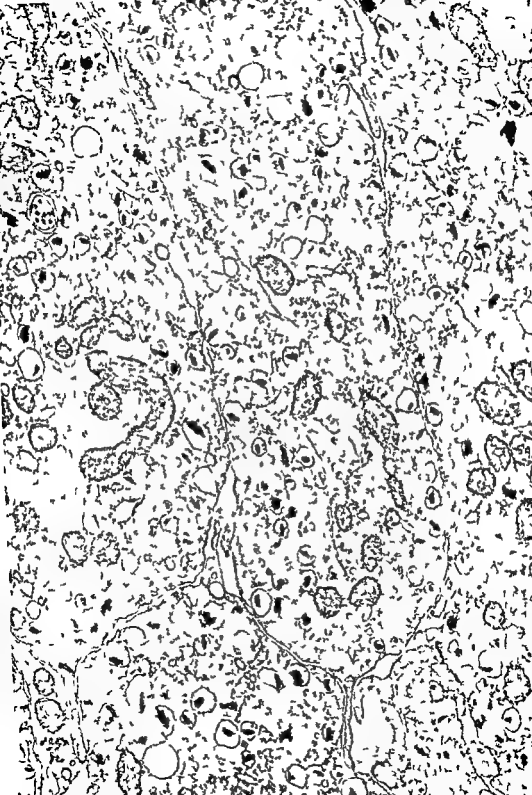


*Fig 5 Fluorescence microscopic picture of paraganglion from a newborn rabbit 170 $\times$  Intensely fluorescent cells throughout the entire parenchyma*

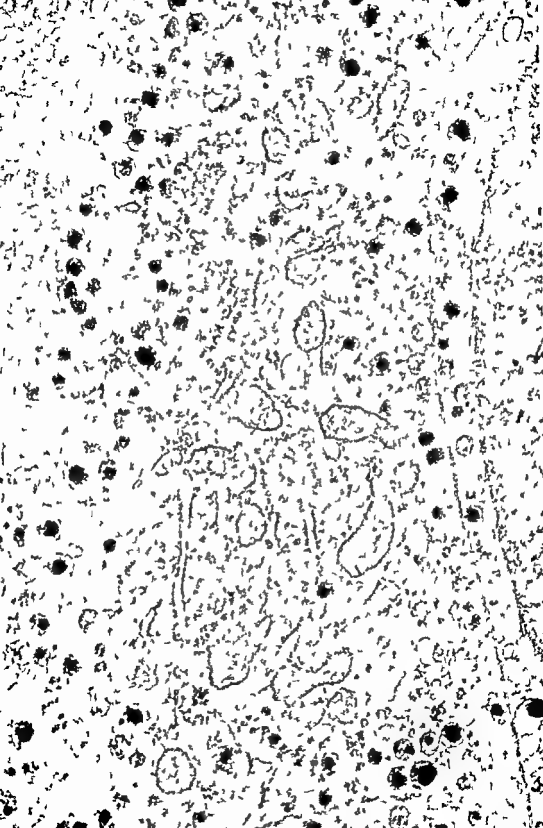


*Fig 6 Fluorescence microscopic picture of paraganglion from a 10 month old rabbit 170 $\times$  Only few islets of fluorescent cells remain*









1965 b, Brundin, Hamberger and Norberg 1966) where NA and A in paraganglia of rabbits have been measured without preceding separation of the amines. Low values for A have been obtained in most but not in all cases. These A values could possibly have been due to calculation artefacts which may occur when measuring NA and A in extracts with large differences between the concentrations of the two amines. However the present results indicate that NA is the only CA of reasonable importance for a possible physiologic role of the paraganglia as CA storing organs. Therefore the paraganglia have been analyzed only for NA in the quantitative studies.

The parallel determinations of CA in both the adrenal glands and the paraganglia of fetal rabbits revealed a marked qualitative difference between the CA contents in the two organs. Thus in the adrenal glands A was the predominant CA whereas in the paraganglia NA was the only CA demonstrable. This distinct difference seems remarkable in view of the close anatomical relationships between these two organs. However the capacity of methylating NA to A in the adrenal glands is not necessarily an inherent property of the adrenomedullary cells. An influence of extramedullary factors upon the hormone content of the adrenal medulla has been suggested earlier by several authors (Shepherd and West 1951, Hokfelt 1951 and others). Recently it was reported that hypophysectomy in rats caused a reduction of the adrenal content of the NA methylating enzyme phenylethanolamine *N*-methyl transferase and that this reduction could be prevented by the administration of either ACTH or glucocorticoid hormone (Wurtman and Axelrod 1965). Moreover in the paraganglionic tissue of rats where normally NA is the only CA present A has been demonstrated after pretreatment with corticosteroids (Eranko, Iempinen and Karsanen 1966) — Thus the presence of corticosteroids seems to be required for the formation of A in the chromaffin cell. In the adrenal medulla high concentrations of corticosteroid hormones could easily be supplied via the direct vascular connections which have been found between the cortex and the medulla (for ref. see Waller 1955). Since no

*Fig. 7* Survey picture of paraganglionic cells from a newborn rabbit 15 000  $\times$ . Numerous osmiophilic granules and mitochondria are scattered in the cytoplasm.

*Fig. 8* Paraganglionic cells from a newborn rabbit 36 000  $\times$ . Most granules exhibit small electron dense cores of irregular shape and large pale peripheries. Certain obliquely cut granules exhibit no electron dense material. Free and membrane bound ribosomes frequently occur in the cytoplasm. A cross section of cilium with 8 + 1 double filaments is seen in the upper left part.

*Fig. 9* Adrenal cells from a newborn rabbit 36 000  $\times$ . In the medullary cells the cytoplasm contains numerous osmiophilic granules and mitochondria. The granules show relatively large rounded electron dense cores, the peripheries being dotted and of a certain opacity. Layers of thin cellular sheets separate medullary and cortical cells (upper left part).

such vessels from the adrenocortex have been demonstrated in the paraganglia the absence of A in the paraganglia is presumably a consequence of insufficient direct adrenocortical influence

In the *foetus* near term the paraganglia contained about twice as much CA as the adrenal glands. The gradually increased NA content in the paraganglia observed during the first postnatal week might reflect a progressive functional development of the storage and/or the synthesis of NA in the organ. The considerably increased NA content observed already on the first postnatal day might be explained by a sudden acceleration of this development at birth. On the other hand, if the paraganglia were able to release NA continuously during fetal life and this release was interrupted at birth a sudden increase of the organ content of NA could also occur (*cf* Chapter V)

The small individual variations of the NA content in the paraganglia of one and two day old rabbits indicate that animals of this age ought to be the most suitable for comparative studies on the paraganglia. This condition was also exploited in the functional studies (Chapter IV and V)

From the fifth postnatal day the individual values showed wider variations within the groups. At four weeks after birth the mean NA content of the paraganglia was considerably decreased even below the level for the fetal organs studied. This increased variation and final reduction of the NA contents indicate a functional degeneration of the NA storing capacity of the paraganglia

In the histochemical studies the parenchyma of the paraganglia from newborn rabbits was found to consist entirely of cells with large CA contents. The appearance of connective and fatty tissues at the expense of the CA containing cells in the paraganglia of two month old rabbits further corroborated that the organs undergo a postnatal degeneration. — The earlier concept that the paraganglia are permanent organs in the rabbit (see Chapter I) was based on the observation that the chromaffin reaction was demonstrable also in the paraganglia of adult animals. Even though the organ thus might persist in the adult rabbit the present results evidently show that degenerative changes impair the CA storing properties of the paraganglia soon after birth

In the differential centrifugation studies on homogenates of paraganglia and adrenal glands the main portion of the total organ contents of CA was found in the high speed sediments. The proportions between sedimentable and non sedimentable CA in the paraganglia were similar to those in the adrenal glands. The rate of spontaneous release of CA from the sedimentable fraction of paraganglionic homogenates was measured *in vitro* in a special study

(Brundin 1966) Also in this respect the paraganglia were similar to the adrenal glands — It seems likely that the sedimenting CA were bound to a granular microsomal fraction in these organs as well as in other CA storing tissues (*cf* Chapter 1) It has been shown, however that supernatants obtained by centrifugation of organ homogenates at  $1\,000 \times g$  contain both the microsomal and mitochondrial fractions of the cells (for ref see Mathias 1966) Therefore, it could not be excluded that some of the CA in the present high speed sediments were bound to other structures than granules On the other hand, since the electron microscopic studies revealed very large amounts of osmiophilic granules in the cytoplasm of both the paraganglionic and adrenomedullary cells from the newborn rabbits, it is reasonable to assume that the major portion of the CA stores was associated with the granular fraction of the cells

In the electron microscope the paraganglionic granules differed characteristically from those of the adrenal medulla in that their electron dense cores were considerably smaller and of irregular shape As judged from the mitochondria in the same sections no general swelling artefact was responsible for this appearance of the paraganglionic granules The paraganglia as well as the adrenal glands were obtained from the same animals and treated identically Thus the paraganglionic granules were different from the adrenomedullary ones The difference observed in the sections might be due to a different reaction to the preparation procedure — The numerous mitochondria observed in the paraganglionic cells indicate that energy consuming processes occur in the cells The large amounts of ribosomes might be correlated to a high capacity of protein synthesis — Cilia have frequently been observed on cells derived from the neural crest (Palay 1961 Dahl 1963 and others) On the chromaffin cells of the adrenal medulla of adult rabbits cilia have been demonstrated (De Robertis and Sabatini 1960) In the present study cilia were observed on paraganglionic cells This might be a sign of the neural origin of the organ

It is concluded that the preaortal paraganglia are large sources of NA in fetal and newborn rabbits and that the organs undergo a postnatal degeneration soon after birth In the newborn rabbit the storage of CA in the paraganglia resembles that in the adrenal medulla in many respects Thus the bulk of the CA stores in the paraganglia is bound to subcellular structures and large amounts of osmiophilic granules are demonstrable in the cytoplasm of paraganglionic cells Even though the appearance of these granules in the electron micrographs is characteristically different from that of adrenomedullary granules many features of the general ultrastructure are similar in the two organs

STUDIES ON THE FORMATION OF CATECHOL  
AMINES IN THE PARAGANGLIA

In order to study the synthesis of NA in the paraganglionic tissue the incorporation of radioactive CA was measured in the paraganglia from newborn rabbits pretreated with  $C^{14}$  labeled CA precursors. The adrenal glands from the same animals were used as reference organs. The uptake of injected radioactive NA in the paraganglia was also studied.

## Special methods

Incorporation of radioactive catecholamines from  $C^{14}$  labeled precursors

Each of 8 newborn rabbits from the same litter received *dl* DOPA  $2\ C^{14}$  ( $262\ \mu\text{C}/\mu\text{mole}$ ) in a single s.c. injection of  $0.12\ \mu\text{C}/\text{g b.w.}$  (total dose of DOPA  $9\ \mu\text{g}/\text{g b.w.}$ ). The volumes injected were about 1 ml. At different intervals after the injections (10, 30 and 60 min, 24 and 36 hrs) the animals were decapitated and their paraganglia and adrenal glands were excised and extracted. The CA of the extracts were then separated by column chromatography, the paraganglionic extracts on 5 cm, the adrenal extracts on 15 cm Amberlite columns. Unlabeled DA was added to the extracts before the separations in order to localize this amine in case radioactivity should appear in these fractions. The direct fluorescence and the radioactivity were measured in each fraction of the chromatograms (for details see General methods).

To another litter of 6 newborn rabbits *L*-tyrosine  $C^{14}$  uniformly labeled ( $351\ \mu\text{C}/\mu\text{mole}$ ) was given in single s.c. injections of  $0.3\ \mu\text{C}/\text{g b.w.}$  (total dose of tyrosine  $0.15\ \mu\text{g}/\text{g b.w.}$ ). The paraganglia and adrenal glands of these animals were excised at 1, 4, 12, 24, 48 and 72 hrs after the injection of tyrosine  $C^{14}$ . The separation and analysis of CA were performed as described above.

---

\* The radioactive chemicals were obtained from New England Nuclear Corp.

## Uptake of $H^3$ labeled noradrenaline

To one newborn rabbit NA  $7 H^{38}$  ( $8.1 \text{ mCi}/\mu\text{mole}$ ) was injected in a dose of  $0.15 \text{ } \mu\text{Ci/g bw}$  (total dose of NA  $4 \text{ ng/g bw}$ ). At 35 min after the injection the animal was killed and the paraganglion, the adrenal glands and the heart were excised. The organs were extracted with  $2 \text{ ml } 0.4 \text{ M}$  perchloric acid. After centrifugation  $500 \text{ } \mu\text{l}$  of each extract was measured for radioactivity.

## Results

### Incorporation of $C^{14}$ labeled catecholamines after pretreatment with dihydroxyphenylalanine $C^{14}$

The incorporation of radioactive CA in the paraganglia and adrenal glands is presented in Fig. 10. The total radioactivity values of each CA peak are listed in Table V.

Only small amounts of radioactivity were found in the organs excised 10 min after the injection of DOPA  $C^{14}$ . — At 30 min after the pretreatment, peaks of radioactive NA were found in both the paraganglionic and the adrenal chromatograms. In the paraganglia the radioactivity of the NA fractions gradually increased during the whole period studied.

In the adrenal glands radioactive NA was rapidly incorporated. The maximum peak was obtained 4 hrs after the pretreatment. After 24 and 36 hrs only small amounts of NA  $C^{14}$  were present in the adrenal glands. — Radioactive A appeared in the adrenal chromatograms but it was not until 4 hrs after the DOPA  $C^{14}$  injection that a proper peak of A  $C^{14}$  was found. However, at 24 and 36 hrs after the injection most of the adrenal radioactivity was confined to the A fractions.

Small amounts of radioactivity were sometimes observed in the DA fractions of the organs removed within one hour after the injection of DOPA  $C^{14}$  but no proper peak of DA  $C^{14}$  was found in any of the chromatograms.

The molar quantities of radioactive CA in the organs were calculated from the values in Table V. A summary of the results (Fig. 11) shows that the incorporation as well as the spontaneous disappearance of NA  $C^{14}$  occurred at a considerably lower rate in the paraganglia than in the adrenal glands.

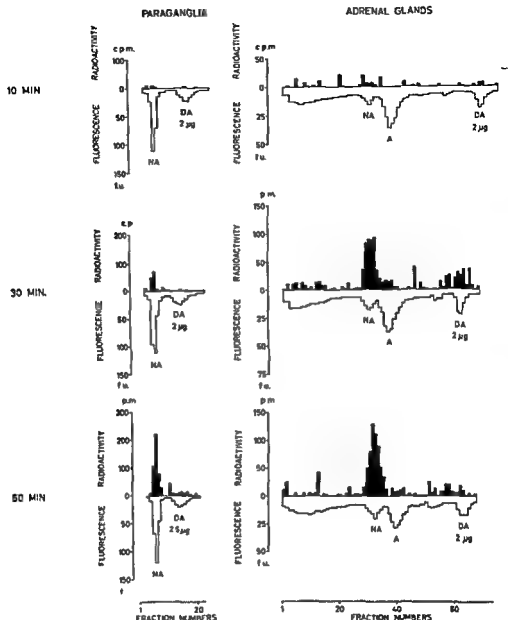
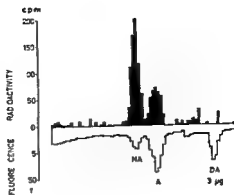
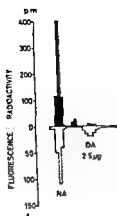


Fig 10 Chromatograms showing the incorporation of radioactivity in extracts of paraganglia and adrenal glands of newborn rabbits at different intervals after the administration of DOI-AC. DA was added to all the extracts. Separation of CA on 5 cm columns for paraganglia and 10 cm columns for adrenal extracts.

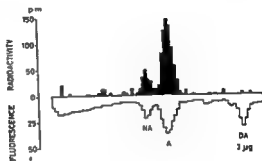
# PARAGANGLIA

# ADRENAL GLAND

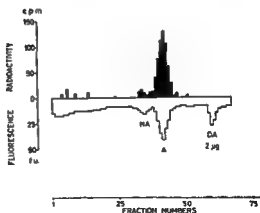
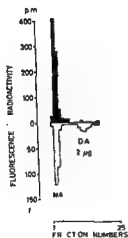
HOURS



4 HOURS



36 HOURS

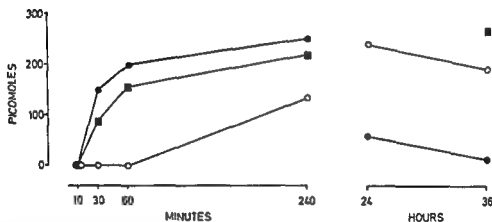




**Table I** Radioactivity (cpm) within the CA containing peaks from the chromatograms of paraganglia and adrenal glands at different intervals after injection of DOPA  $C^{14}$

Intervals after DOPA $C^{14}$ inj	PARAGANGLIA	ADRENAL GLANDS	
	NA	NA	A
10 min	<100	(119)*	<100
30 >	125	123	<100
60 >	444	570	<100
1 hrs	630	690	388
24 >	—	171	696
36 >	466	<100	521

\* This radioactivity lacked the characteristic peak formed distribution in the chromatogram



**Fig 11** Total amounts of  $C^{14}$  labeled CA recovered from paraganglia and adrenal glands of new born rabbits at different intervals after administration of DOPA 2  $C^{14}$  0.17  $\mu$ C/g bw

■—■ NA in paraganglia  
●—● NA in adrenal glands  
○—○ A in adrenal glands

## Incorporation of $C^{14}$ labeled catecholamines after pretreatment with tyrosine $C^{14}$

The incorporation of radioactive CA in the paraganglia and adrenal glands is presented in Fig 12 and in Table VI

In the paraganglion excised one hour after the injection of tyrosine  $C^{14}$  only small amounts of radioactivity were found in the NA fractions. However, at all the subsequent intervals studied well defined peaks of radioactivity were observed in the NA fractions of the paraganglia. The maximum amount of radioactive NA was obtained at 24 hrs after the pretreatment and at 48 and 72 hrs the radioactive peaks were reduced in the paraganglionic chromatograms.

In the adrenal glands a clearcut peak of radioactive NA was found already one hour after the pretreatment. At this early stage the adrenal chromatogram was to some extent contaminated by unspecific radioactivity and no proper peak of A  $C^{14}$  was observed. At 4 hrs after the tyrosine  $C^{14}$  injection a peak of radioactive A had appeared. At the subsequent intervals the amounts of NA  $C^{14}$  decreased in the adrenal glands and the main contents of radioactivity were confined to the A fractions. After 72 hrs the adrenal chromatogram was not free from contamination and the peaks of labeled CA were relatively small.

DA  $C^{14}$  was not measured in the adrenal glands excised one hour after the pretreatment. In the other chromatograms no radioactivity was found in the DA fractions.

The molar quantities of radioactive CA were calculated from the values in Table VI. A summary of the results (Fig 13) shows that the maximum amounts of labeled CA were higher in the paraganglia than in the adrenal glands. However the paraganglia required more time for the accumulation of significant amounts of NA  $C^{14}$  and the spontaneous disappearance of radioactive CA was slower from the paraganglia than from the adrenal glands.

## Uptake of noradrenaline

The uptake of NA in the paraganglia was evaluated by measuring the radioactivity in the organ at 35 min after an injection of  $H^3$  labeled NA. The radioactivity was also measured in the adrenal glands and the heart for comparison. The amounts of radioactivity in the paraganglion (338 cpm) and the adrenal glands (208 cpm) were very low as compared to that in the heart (7096 cpm).

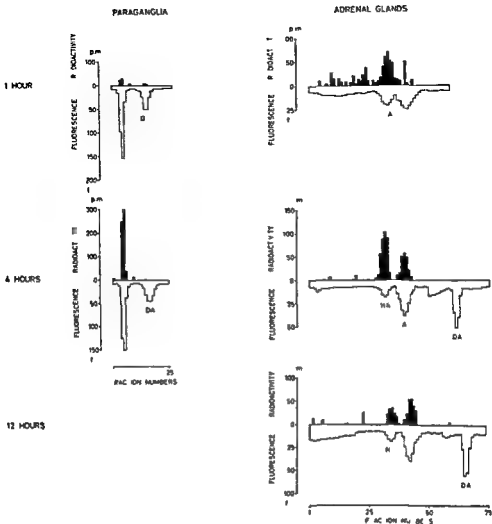
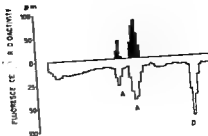
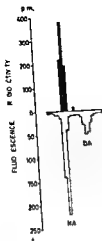


Fig 17 Chromatograms showing the incorporation of radioactivity in extracts of paraganglia and adrenal glands of newborn rabbits at different intervals after the administration of tyrosine C<sup>1</sup>. DA was added to all extracts except the first adrenal. Separation of CA on 5 cm column for paraganglionic extracts on 15 cm columns for adrenal extracts

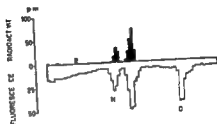
# PARAGANGLIA

# ADRENAL GLANDS

5 HOURS



12 HOURS



22 HOURS

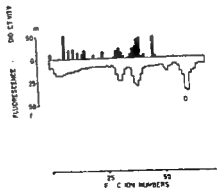
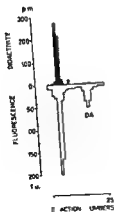


Table 11 Radioactivity (cpm) within the C1 containing peaks from the chromatograms of paraganglia and adrenal glands at different intervals after injection of tyrosine C<sup>14</sup>

Hours after tyrosine C <sup>14</sup> inj	PARAGANGLIA	ADRENAL GLANDS	
	NA	NA	A
1	<100	302	<100
4	609	375	290
12	—	115	295
24	797	<100	325
48	313	<100	170
72	391	<100	152

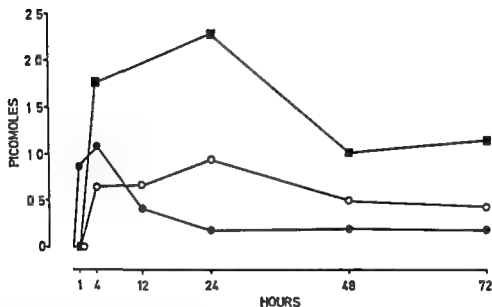


Fig. 13 Total amounts of C<sup>14</sup> labeled CA recovered from paraganglia and adrenal glands of newborn rabbits at different intervals after administration of tyrosine C<sup>14</sup> (uniformly labeled): 0.3  $\mu$ C/g b.w.

■—■ NA C<sup>14</sup> in paraganglia  
 ●—● NA C<sup>14</sup> in adrenal gland  
 ○—○ A C<sup>14</sup> in adrenal glands

## Discussion and conclusions

In two experimental series the incorporation of radioactive NA in the paraganglia was studied *in vivo* at different intervals after the administration of  $C^{14}$  labeled CA precursors. For comparison the adrenal glands were studied as well — Principally the same technique of CA separation was used for the different organs but the adrenal extracts required a modification of the method for the separation of NA from A. The influence of this modification upon the recovery for CA was small in the actual chromatograms as judged from the fluorescence peaks and did not prevent comparative studies of the two organs — Due to the different specific activities of the two precursors used the incorporation of radioactive CA was studied at considerably different quantitative levels in the two series of experiments.

The fact that the uptake of injected radioactive NA was very low in both the paraganglia and the adrenal glands indicates that the radioactive CA found in the organs after the administration of the precursors had been synthesized within the organs.

The question whether the paraganglionic tissue, like the adrenal medulla, is able to perform all the intermediate conversions in the synthetic pathway from tyrosine requires *in vitro* experiments for elucidation. In the present *in vivo* study the incorporation of radioactivity was studied in only one of the intermediate products namely DA. — In adult mice  $C^{14}$  labeled DA has been found in the adrenal glands after the administration of DOPA  $C^{14}$  (Rosell Sedvall and Ullberg 1963) and it was suggested that the precursor partly was taken up by the adrenal medulla in the decarboxylated form. — In the present experiments no peaks of radioactive DA were obtained at any of the time intervals studied neither in the paraganglionic nor in the adrenal chromatograms. Even though this failure to demonstrate DA  $C^{14}$  partly could have been due to the relatively low recovery for this amine (see General methods) it might indicate that the conversion of DA to NA in these organs occurred too rapidly to permit an accumulation of DA.

After the administration of either DOPA  $C^{14}$  or tyrosine  $C^{14}$  the maximum amounts of radioactive NA recovered from the paraganglia exceeded those recovered from the adrenal glands. However the paraganglionic NA peaks never reached the high specific activity found in the corresponding adrenal peaks (*cf.* the low total NA content in the adrenal glands). This could indicate that the capacity of NA synthesis was lower in the paraganglia than in the adrenal medulla. — The fact that in both the experimental series NA  $C^{14}$  appeared later in the paraganglia than in the adrenal glands strongly suggests that the rate of NA formation is lower in the paraganglia than in the adrenal medulla.

The relatively slow spontaneous disappearance of  $NA-C^{14}$  from the paraganglia might further support this suggestion. On the other hand, a direct quantitative comparison of the rates of the spontaneous disappearance of  $NA-C^{14}$  from the different organs may be misleading, since it can not be excluded that some of the adrenal  $NA-C^{14}$  represented an intermediate step in the A synthesis and thus disappeared due to methylation. However, in the long term experiment at 72 hrs after the injection of tyrosine  $C^{14}$  the amounts of  $NA-C^{14}$  remaining in the paraganglia exceeded even the sum of the  $C^{14}$  labeled NA and A in the adrenal glands. Thus in this experiment the replacement of the radioactive CA was slower in the paraganglia than in the adrenal glands, suggesting a relatively lower turnover rate in the paraganglia.

It is concluded that the paraganglia are able to synthesize NA *in vivo*. In spite of the comparatively low rate of the NA synthesis in the paraganglia, the relatively large quantities of NA formed by the organs are probably of importance for the physiology of the newborn animal.

## EFFECTS OF CELLULAR GLUCOPENIA AND ASPHYXIA UPON THE CATECHOLAMINE CONTENTS IN PARAGANGLIA AND ADRENAL GLANDS

*It has been shown in studies on adult animals that the dramatic release of CA from the adrenal glands after insulin induced hypoglycemia leads to a reduction of the total adrenal content of CA (Burn, Hutcheon and Parker 1950, Hokfelt 1951 and others). Similar effects on the adrenal glands have been demonstrated in animals subjected to cellular glucopenia by the administration of 2 deoxy glucose (2 DG) (Hokfelt and Bygdeman 1961) — In fetal and newborn animals the adrenal glands have been found to release large amounts of CA during asphyxia (for ref. see Chapter I page 10)*

A study was undertaken in order to find out whether the paraganglionic CA content was affected by experimentally induced cellular glucopenia or asphyxia. For comparison the adrenal glands were studied simultaneously.

### Special methods

#### Cellular glucopenia

Two litters of 6 newborn rabbits each were used. To 3 animals of each litter a 5.5 % aqueous solution of 2 DG was injected s.c. in two doses of 0.5 mg/kg b.w. each and with an interval of 5 hrs between the injections. The remaining animals served as controls and received corresponding volumes of saline s.c. During the experiments the animals were kept apart from their mothers and were not fed with milk or water. At 5 hrs after the last injections the animals were sacrificed. The paraganglia and adrenal glands were excised and their CA contents were determined fluorimetrically.

Cellular glucopenia was similarly induced in 3 specimens from a litter of 6 two day old rabbits which were all pretreated with DOPA 2 C<sup>14</sup>, 0.12  $\mu$ C/g b.w. 33 hrs before. The remaining 3 animals were used as controls. At 5 hrs after the last injections of 2 DG the animals were decapitated. Their paragang



ionic contents of NA isolated chromatographically were measured for radioactivity (for details see General methods)

## Asphyxia

Three litters of 2, 4 and 6 newborn rabbits respectively were used. Each litter was divided in an experimental and a control group. The 6 experimental animals were subjected to asphyxia by enclosure in stoppered plexi glass tubes of 100 ml volume. The tightness of the tubes was tested by submersion in water. After 10 min in the tubes the animals were let out and allowed to recover for 10 min. After an additional 10 min period in the tubes the animals were decapitated and their parathyroid and adrenal glands were excised and analyzed for CA.

## Hypoxia

Two litters of 6 newborn rabbits each were used. Three animals of each litter served as controls. The other animals were subjected to hypoxia by enclosure for 40 min in plexi glass tubes filled with a gas mixture of 5% O<sub>2</sub> in N<sub>2</sub>. Every second min the gas mixture was blown through the tubes for 30 sec. After a 10 min interval of recovery the procedure was repeated for another 10 min period after which both the experimental and control animals were killed. Their parathyroids were removed and analyzed for CA.

## Hypercapnia

In a corresponding manner hypercapnia was induced by a similar technique but the gas mixture used was 15% CO<sub>2</sub> in O<sub>2</sub>.

## Statistical methods\*

The results were treated according to ordinary methods for analysis of variance (for ref. see Snedecor 1956).

\* For assistance with the statistical calculations I am greatly indebted to B. Lindström at the Department of Medical Physics, Karolinska Institutet.

## Results

### Effects of cellular glucopenia upon the catecholamines in paraganglia and adrenal glands

The effects of 2 DG administration upon the total contents of CA in the adrenal glands and paraganglia are presented in Table VII. In the adrenal glands the CA content decreased significantly after cellular glucopenia. The proportions of NA in the organs varied from 20 to 33 % and were not changed by the treatment. In the paraganglia the NA content was not significantly changed but showed a slight increase in the 2 DG treated animals.

In order to find out whether the paraganglionic synthesis of NA was affected by cellular glucopenia, 2 DG was given to 3 of 6 animals in the paraganglia of which  $C^{14}$  labeled NA was previously incorporated by pretreatment with DOPA- $C^{14}$ . The total amounts of NA- $C^{14}$  in the paraganglia were not changed by cellular glucopenia (Table VIII).

*Table VII Effects of 2 DG induced cellular glucopenia upon the CA contents of adrenal glands ( $\mu\text{g}/\text{pair of organs}$ ) and paraganglia ( $\mu\text{g}/\text{organ}$ ) in 2 litters of newborn rabbits*

Litter	ADRENAL GLANDS				PARAGANGLIA	
	Glucopenia		Controls		Glucopenia	Controls
	NA+A	% NA	NA+A	% NA	NA	NA
a	3.91	27	6.44	25	8.67	7.23
	4.43	25	5.03	21	6.90	5.79
	3.57	26	6.75	26	9.21	6.03
Means	3.97	26	6.07	24	8.26	6.35
b	3.59	29	5.48	33	7.97	6.27
	4.10	29	6.01	20	6.34	6.04
	4.27	20	5.99	28	9.03	8.76
Means	3.99	26	5.83	27	7.75	6.86
Difference Controls—Glucopenia $P < 0.001$				Difference Glucopenia—Controls $P > 0.1$		

honic contents of  $\text{N}_2$  isolated chromatographically were measured for radioactivity (for details see General methods)

### Asphyxia

Three litters of 2, 4 and 6 newborn rabbits respectively were used. Each litter was divided in an experimental and a control group. The 6 experimental animals were subjected to asphyxia by enclosure in stoppered plexi glass tubes of 400 ml volume. The tightness of the tubes was tested by submersion in water. After 10 min in the tubes the animals were let out and allowed to recover for 10 min. After an additional 10 min period in the tubes the animals were decapitated and their parathyroid and adrenal glands were excised and analyzed for  $\text{Ca}^{45}$ .

### Hypoxia

Two litters of 6 newborn rabbits each were used. Three animals of each litter served as controls. The other animals were subjected to hypoxia by enclosure for 40 min in plexi glass tubes filled with a gas mixture of 3%  $\text{O}_2$  in  $\text{N}_2$ . Every second min the gas mixture was blown through the tubes for 30 sec. After a 10 min interval of recovery the procedure was repeated for another 10 min period after which both the experimental and control animals were killed. Their parathyroids were removed and analyzed for  $\text{Ca}^{45}$ .

### Hypercapnia

In a corresponding material hypercapnia was induced by a similar technique but the gas mixture used was 15%  $\text{CO}_2$  in  $\text{O}_2$ .

### Statistical methods\*

The results were treated according to ordinary methods for analysis of variance (for ref. see Snedecor 1956)

\* For assistance with the statistical calculations I am greatly indebted to H. Lindstrom at the Department of Medical Physics, Karolinska Institutet.

*Table 1 Effects of hypoxia and hypercapnia upon the total contents of NA ( $\mu$ g) in the paraganglia of newborn rabbits*

Litter	Hypoxia	Controls	Litter	Hypercapnia	Controls
a	4.90	8.15	c	4.10	8.14
	6.45	7.90		3.53	7.35
	6.20	7.37		1.50	7.08
b	7.47	6.40	d	5.35	6.35
	6.03	5.85		5.82	5.90
	5.70	7.10		6.80	6.20
<i>Difference</i> Controls—Hypoxia P>0.1				<i>Difference</i> Controls—Hypercapnia P>0.1	

reduced in those animals subjected to asphyxia. In the adrenal glands of the same animals the CA contents were not significantly changed. Nor did the treatment affect the proportions of the adrenal NA content.

In order to perform a more detailed study on the effects of asphyxia upon the paraganglionic NA content, two additional experimental series were undertaken in which the NA content was measured in the paraganglia of newborn rabbits subjected to either hypoxia or hypercapnia. The paraganglionic contents of NA after these treatments are listed in Table 1. In two litters the values decreased considerably after hypoxia and hypercapnia alone, but in two other litters no overt changes occurred.

### Discussion and conclusions

It is well known from the literature that certain pathophysiological conditions such as hypoglycemia and asphyxia stimulate the release of CA from the adrenal medulla (Cannon and Hoskins 1911; Burn, Hutcheon and Parker 1950; Hokfelt 1951 and others). — The effects of hypoglycemia (and of cellular glucopenia following the administration of 2 DG) upon the adrenal CA release are mediated via the splanchnic nerves and can be abolished by denervation of the adrenal glands (Hokfelt and McLean 1950; Hokfelt and Bygdeman 1961). Asphyxia, on the other hand, has been found to increase the release of CA also from the denervated adrenal medulla. This effect of asphyxia seems to be most easily demonstrable in fetal and newborn animals. Thus in lambs and calves

subjected to asphyxia Comline and Silver (1958, 1961) found considerably augmented concentrations of CA in the blood drawn from a pouch of *vena cava* at the level of the adrenal veins. In these experiments the NA content of the blood was much more increased by asphyxia than was the A content. Although this result was interpreted as a sign of increased adrenomedullary activity it was recently suggested that a secretion of NA from the paraganglionic tissue might have reinforced the effect obtained (Comline and Silver 1966). However, no evidence was presented for the existence of such a secretory response from the paraganglia.

An accurate estimation of the release of CA from the adrenal glands (or from the paraganglia) would require analysis of the CA contents in the venous blood from the organs. Since it is technically impossible to catheterize the paraganglionic or the adrenal veins of the newborn rabbit it is not feasible to perform a proper quantitative study of the CA release from these organs. — However it is known from studies on adult animals that the release of large amounts of CA from the adrenal medulla is regularly accompanied by a depletion of the CA stores in the organ. In consequence a significantly reduced CA content in the adrenal glands indicates that a considerable amount of CA has been released from the organs. — This ought to be valid also for the paraganglia. In view of the relatively slow synthesis of CA in these organs a release of significant amounts of CA should readily be reflected by a deficit in the paraganglionic CA stores. — In the present experiments the release of CA from the paraganglia and adrenal glands was studied in newborn rabbits by measuring the effects of cellular glucopenia and of asphyxia upon the total organ contents of CA.

The fact that the adrenal CA content decreased significantly after cellular glucopenia indicates that the nervous supply to the adrenal medulla is developed up to a functional state already in the newborn rabbit. In the paraganglia the CA content was not decreased by cellular glucopenia, but showed, on the contrary, a remarkable tendency to increase. The mechanism for this slight increase seems difficult to explain. There was no increased rate of NA formation in the paraganglia of those animals subjected to glucopenia since the replacement of previously incorporated NA- $C^{14}$  by unlabeled NA was not changed in the paraganglia of these animals. These results evidently show that the paraganglia do not release CA parallel to the adrenal medulla. Moreover, the present results support the statement by Muscholl and Vogt (1964) that the paraganglia lack a functional innervation via the splanchnic nerves. This lack of innervation might partly explain the relatively low rate of NA synthesis in the paraganglia (cf. Kroneberg and Schuman 1959).

After asphyxia there was a significant reduction of the NA content in the paraganglia. Thus asphyxia elicited a release of NA from the paraganglionic

tissue. The mechanism for this asphyxia induced release from the paraganglia might be similar to that for the release of CA from the denervated adrenal medulla demonstrated in asphyctic limbs and calves by Comline and Silver. In the present experiments, however, the CA contents of the adrenal glands did not change significantly. There was a slight decrease of the total adrenal CA content and the ratio of NA/A was essentially unchanged in the organs.

Since asphyxia is a rather multiform and unspecific method of stimulation, an attempt was made to separate the effects upon the paraganglionic NA content of the two main components of asphyxia: hypoxia and hypercapnia. Both after hypoxia and hypercapnia the paraganglionic NA contents decreased considerably in certain litters, but these effects could not be consistently reproduced.

Even though the asphyxia induced was rather severe, it can not be excluded that also more moderate changes of  $pO_2$  and  $pCO_2$  could exert a stimulation of the NA release from the paraganglia. In fact the comparatively low NA content found in the paraganglia of rabbit *foetus* at full term could reflect a deficit of the NA stores due to such a continuous release from the organs.

It is concluded that the paraganglia do not give any secretory response to cellular glucopenia and that they lack a functional innervation. Asphyxia stimulates the release of NA from the paraganglia probably by a direct action upon the paraganglionic cells. The functional role of this asphyxia induced NA release has not been specially studied but it seems likely that this is an important function of the paraganglia, not least in view of the common clinical problem fetal asphyxia.

## SUMMARY

The present work was undertaken in order to investigate the pre-natal paraganglia of newborn rabbits with respect to their storage, synthesis and release of catecholamines. In most experiments the adrenal glands from the same animals were simultaneously studied for comparison.

The catecholamine stores in the paraganglia were found to consist of noradrenaline exclusively. Quantitatively, the paraganglionic catecholamine content far exceeded that of both the adrenal glands during the perinatal period. Histochemical studies showed that in the newborn rabbit the parenchyma of the paraganglion entirely consisted of catecholamine containing cells, whereas in the two month old rabbit the organ was degenerated by fibrosis. This post-natal degeneration was accompanied by a reduced organ content of noradrenaline. — The subcellular distribution of noradrenaline in the paraganglia was studied by means of differential centrifugation of organ homogenates. About 70 % of the total organ content of noradrenaline was bound to sedimentable structures. A similar ratio of sedimentable/non sedimentable catecholamines was found in adrenal homogenates. — Electron microscopical studies revealed that the ultrastructure of the paraganglia in many respects resembled that of the adrenal medulla. Large amounts of cytoplasmic osmiophilic granules were found in both the organs. The appearance in the electron micrographs of the paraganglionic granules differed from that of the adrenomedullary ones.

The synthesis of noradrenaline *in vivo* was found to be comparatively slow in the paraganglia. After the administration of  $C^{14}$  labeled tyrosine or dihydroxyphenylalanine both the incorporation and the subsequent spontaneous disappearance of radioactive noradrenaline occurred at lower rates in the paraganglia than in the adrenal glands.

The release of catecholamines from the paraganglia was measured indirectly by determination of the reduction of the organ content of noradrenaline in animals subjected to experimentally induced stressful situations. Cellular glucopenia following the administration of 2 deoxyglucose reduced the adrenal catecholamine content significantly but did not affect the noradrenaline stores of the paraganglia. Since the effects of cellular glucopenia upon the adrenal medulla are mediated via the splanchnic nerves the present results support the opinion of previous authors that the paraganglia probably lack a functional

innervation — Severe asphyxia elicited a release of noradrenaline from the paraganglia reflected by a significant reduction of the noradrenaline content of the organs. No reproducible significant changes were observed after separately induced hypoxia or hypercapnia.

It is suggested that the paraganglia are of functional importance during the perinatal period especially under the common pathophysiological condition fetal asphyxia.



## ACKNOWLEDGMENTS

To Professor U S von Euler under whose guidance I have had the great privilege to work I wish to express my sincere gratitude for his stimulating advice and constructive criticism

My thanks also go to Dr SFG Nilsson Dr K A Norberg and Dr H Hamberger for pleasant collaboration and generous help throughout the electron microscopic and histochemical studies and to Dr H Lindstrom for helpful assistance with the statistical calculations

My thanks are also due to Mrs K Lemon for reviewing the English text and to all the members of the Department of Physiology for fruitful discussions and helpful interest in my work

This investigation was supported by research grants from Karolinska Institutet Magnus Bergwalls Stiftelse Svenska Sällskapet for Medicinsk Forskning Stiftelsen Lars Hierras Minne Expressens Prenatalforskningsfond and by grants to Professor v Euler from the AIR FORCE OFFICE OF SCIENTIFIC RESEARCH under Grant AF EOAR 66-7 through the European Office of Aerospace Research (OAR) United States Air Force which are all gratefully acknowledged

## REFERENCES

- ASDELL E V Patterns of mammalian reproduction Comstock publishing Co Inc Ithaca N Y 1946
- BIEDL A and J WIESEL Ueber die functionelle Bedeutung der Nebenorgane des Sympathicus (Zuckerkanal) und der chromaffinen Zelleruppen *Pflügers Arch ges Physiol* 1907 91 434—461
- BLASCHKE H The specific action of l dopa decarboxylase *J Physiol (Lond)* 1939 96 50 P
- BLASCHKE H and A D WELCH Localization of adrenaline in cytoplasmic particles of the bovine adrenal medulla *Launyn-Schmidberg's Arch exp Path Pharmacol* 1953 219 1—22
- BRUNDIN T Catecholamines in adrenals from fetal rabbits *Acta physiol scand* 1965 a 63 309—310
- BRUNDIN T Catecholamines in the preaortal paraganglia of fetal rabbits *Acta physiol scand* 1965 b 64 287—288
- BRUNDIN T Storage and *in vitro* release rate of catecholamines from granules isolated from preaortal paraganglia and adrenals of newborn rabbits *Acta physiol scand* 1966 66 406—409
- BRUNDIN T and S E G NILSSON Osmophilic granules in preaortal paraganglia from newborn rabbits *Acta physiol scand* 1965 65 287—288
- BRUNDIN T B HANBERGER and K A NORBERG Postnatal changes in the preaortal paraganglia of rabbits *Acta physiol scand* 1966 66 255—256
- BLUM J H D E HUTCHESON and R H O PARKER Adrenaline and noradrenaline in the suprarenal medulla after insulin *Brit J Pharmacol* 1950 5 417—423
- CANNON W B and R G HOSKINS The effects of asphyxia hyperpnoea and sensory stimulation on adrenal secretion *Amer J Physiol* 1911 29 274—279
- CARLSSON A and B Waldeck A fluorimetric method for the determination of dopamine (3 hydroxytyramine) *Acta physiol scand* 1958 44 293—298
- COMLINE R S and M SILVER Response of the adrenal medulla of the sheep foetus to asphyxia *Nature (Lond)* 1958 181 283—284
- COMLINE R E and M SILVER The release of adrenaline and noradrenaline from the adrenal glands of the foetal sheep *J Physiol (Lond)* 1961 156 424—444
- COMLINE R E and M SILVER The development of the adrenal medulla of the foetal and new born calf *J Physiol (Lond)* 1966 183 305—340
- COMLINE R S I A SILVER and M SILVER Factors responsible for the stimulation of the adrenal medulla during asphyxia in the foetal lamb *J Physiol (Lond)* 1963 169 97—98 P
- CORRODI H and V V HILLARF Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 1 Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 67 Dimethoxyisochinolinderivaten und Formaldehyd *Helv chim Acta* 1963 46 2475—2490
- CORRODI H and V A HILLARF Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 2 (1) Identifizierung des fluoreszierenden Produktes aus Dopamine und Formaldehyd *Helv chim Acta* 1964 47 911—918

- COUPLAND R I The development and fate of the abdominal chromaffin tissue in the rabbit *J Anat (Lond)* 1956 90 527—537
- COUPLAND R I The postnatal distribution of the abdominal chromaffin tissue in the guinea pig, mouse and white rat *J Anat (Lond)* 1960 94 241—256
- COUPLAND R I The natural history of the chromaffin cell Longmans Green & Co Ltd London 1965
- DAHL H A Fine structure of cilia in rat cerebral cortex *Zellforsch* 1963 60 369—380
- DR ROBERTS I and D D SABATINI Submicroscopic analysis of the secretory process in the adrenal medulla *Fed Proc* 1960 Suppl 5 70—18
- DOSTOJEWSKY A Ein Beitrag zur mikroskopischen Anatomie der Nebennieren bei Säugethieren *Arch mikr Anat* 1816 27 272—296
- DREYER C I On secretory nerves to the suprarenal capsules *Amer J Physiol* 1899 2 203—219
- DUNER H The influence of the blood glucose level on the secretion of adrenaline and noradrenaline from the suprarenal *Acta physiol scand* 1953 28 Suppl 102
- EURELÉN I Fluorimetric determination of adrenaline II *Farm Rec (Stockh)* 1949 47 242—250
- FRANKO O M JEMPINEN and E RAISANEN Adrenaline and noradrenaline in the organ of Zuckerkindl and adrenals of new born rats treated with hydrocortisone *Acta physiol scand* 1961 66 253—254
- FILIER U S A Noradrenaline Charles C Thomas Publ Springfield Ill 1956
- FILIER U S A and N A HILLARY Evidence for the presence of noradrenaline in submicroscopic structures of adrenergic axons *Nature (Lond)* 1956 177 44—45
- FILIER U S A and T ISHAYKO The estimation of catecholamines in urine *Acta physiol scand* 1959 45 122—131
- FILIER U S A and T ISHAYKO Improved technique for the fluorimetric estimation of catecholamines *Acta physiol scand* 1961 51 318—335
- FILICK B Observations on the possibilities of the cellular localization of monoamines by a fluorescence method *Acta physiol scand* 1962 56 Suppl 197
- FILICK B A A HILLARY G FINKE and A TORP Fluorescence of catecholamines and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962 10 348—354
- FILICK B and C OWMAN A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines *Acta L'eri Lund* 1965 117 1—23
- GREENBERG R E and J I LING Catecholamines in tissues of the human fetus *Pediatrics* 1961 27 904—911
- GUENNE L M Catecholamines and 5-hydroxytryptamine in morphine tolerance and withdrawal *Acta physiol scand* 1963 59 Suppl 204
- GURIN S and A M DELLAVA The biological synthesis of radioactive adrenaline from phenylalanine *J Biol Chem* 1947 170 545—550
- HILLARY N A S LAGERSTEDT and B NILSSON The relation of a granular fraction from the suprarenal medulla containing the sympathomimetic catecholamines *Acta physiol scand* 1953 29 251—253
- HOLLINSHEAD W H The innervation of the abdominal chromaffin tissue *J Comp Neurol* 1937 67 133—149
- HUGGENDAL J On the use of strong exchange resins for determination of small amount of catecholamines *Scand J Clin Lab Invest* 1962 14 337—344
- HORFELT B Noradrenaline and adrenaline in mammalian tissues *Acta physiol scand* 1951 25 Suppl 92

- HOKFELT B and S BIGDEMAN Increased adrenaline production following administration of  $\alpha$  deoxy D glucose in the rat *Proc Soc exp Biol (N Y)* 1961 106 537-539
- HOKFELT B and J MCLEAN The adrenaline and noradrenaline content of the suprarenal glands of the rabbit under normal conditions and after various forms of stimulation *Acta physiol scand* 1950 21 258-270
- KAHN R H Studien an Paraganglien *Pflügers Arch ges Physiol* 1912 147 445-472
- KIRSHNER N Biosynthesis of adrenaline and noradrenaline In *Symp on Catecholamines* held in Bethesda Williams & Wilkins Co Baltimore 1958 350-357
- KOHN A Die Paraganglien *Arch mikr Anat* 1903 62 363-365
- KRONBERG G and H SCHIMMANN Ueber die Bedeutung der Innervation für die Adrenalin synthese im Nebennierenmark *Experientia (Basel)* 1959 15 234-235
- LEIKES Z Der Adrenalinhalt der totalen Nebenniere und des Paraganglion iorticum abdominale des Menschen *Endocrinologie* 1941 23 259-264
- LEMPINEN M Extra adrenal chromaffin tissue of the rat and the effects of cortical hormones on it *Acta physiol scand* 1961 62 Suppl 231
- LEVER J D Electron microscopic observations on the normal and denervated adrenal medulla of the rat *Endocrinology* 1955 57 621-635
- LEVER J D and A C ESTERHILTZ Fine structure of the arteriolar nerves in the guinea pig pancreas *Nature (Lond)* 1961 192 566-567
- LAND A Fluorimetric determination of adrenaline in blood III A new sensitive and specific method *Acta pharmacol (Abh)* 1949 5 231-247
- MATHIAS A P Separation of subcellular particles *Brit med Bull* 1966 22 146-152
- MUSCHOLL E and M VOOT Secretory responses of extramedullary chromaffin tissue *Brit J Pharmacol* 1964 22 193-203
- NIEMINEN K and A PERKKARINEN The noradrenaline and adrenaline content of human fetal adrenal glands and aortic bodies *Ann Med exp Fenn* 1952 30 274-286
- NORBERG K A and B HAMBERGER The sympathetic adrenergic neuron *Acta physiol scand* 1964 63 Suppl 238
- OLIVER G and E A SCHAYER The physiological effects of extracts of suprarenal capsules *J Physiol (Lond)* 1895 18 230-279
- PALAY S L Structural peculiarities of the neuro secretory cells in the preoptic nucleus of the goldfish *Carassius auratus* *Intat Rec* 1961 139 261
- TELLEGRINI E Contributo allo studio della morfologia dell'organo parasimpatico dello Zuckerkandl *Monit ool ital* 1906 17 254-264
- RANCIAPPA K S Cryoscopic effect of separation and concentration of solutes *Nature (Lond)* 1961 191 1087-1088
- ROFFI J Dosage de l'adrenaline et de la noradrenaline dans les surrenales du foetus de lapin au cours de la gestation *J Physiol (Paris)* 1964 56 431-435
- ROSELL S G SEDVALL and S ULLBERG Distribution and fate of dihydroxyphenylalanine  $^{14}C$  (DOPA) in mice *Biochem Pharmacol* 1963 12 265-269
- ROSENFELD G L C LEEFER and S UDENFRIEND Biosynthesis of norepinephrine and epinephrine by the isolated perfused calf adrenal *Arch Biochem* 1958 74 252-265
- SHEPHERD M M and G B WEST Noradrenaline and the suprarenal medulla *Brit J Pharmacol* 1951 6 665-674
- SHEPHERD D M and G B WEST Noradrenaline and accessory chromaffin tissue *Nature (Lond)* 1952 170 42-43
- SNEDECOR G W Statistical method The Iowa State College Press Ames Iowa 1956
- STJARNE L Studies of catecholamine uptake storage and release mechanisms *Acta physiol scand* 1964 62 Suppl 278







ACTA PHYSIOLOGICA SCANDINAVICA  
Vol. 70 Supplementum 291

---

THE REGULATION OF  
PROXIMAL TUBULAR REABSORPTION  
IN THE MAMMALIAN KIDNEY

BY  
PAUL P LEYSSAC

COPENHAGEN  
1966





THE REGULATION OF  
PROXIMAL TUBULAR REABSORPTION  
IN THE MAMMALIAN KIDNEY



ACTA PHYSIOLOGICA SCANDINAVICA

Vol 70 Supplementum 291

---

*From the University Institute for Experimental Medicine  
Copenhagen – Denmark*

THE REGULATION OF  
PROXIMAL TUBULAR REABSORPTION  
IN THE MAMMALIAN KIDNEY

BY  
PAUL P LEYSSAC

COPENHAGEN

1966

The manuscript of the present monograph was submitted to the Faculty of Medicine of the University of Copenhagen June 1965, and was accepted as a doctorate thesis on the 7th of October 1966

*To*  
*Egil Bojesen*



## Previously published papers

- A Leyssac P P Dependence of Glomerular Filtration Rate on Proximal Tubular Reabsorption of Salt  
*Acta physiol scand* 1963 58 236-242
- B Leyssac P P, The In Vivo Effect of Angiotensin on the Proximal Tubular Reabsorption of Salt in Rat Kidneys  
*Acta physiol scand* 1964 62 436-448
- C Leyssac P P, The Effect of Partial Clamping of the Renal Artery on Pressures in the Proximal and Distal Tubules and Peritubular Capillaries in the Rat Kidney  
*Acta physiol scand* 1964 62 449-456
- D Leyssac P P Some Characteristics of the Proximal Tubular Wall Related to Reabsorption During Luminal Occlusion Following Interruption of Glomerular Filtration  
*Acta physiol scand* 1965 63 36-45
- E Leyssac P P, The Luminal Occlusion Time of Proximal Tubules in Young Rats  
*Acta physiol scand* 1965 64 176-181
- F Leyssac, P P The In Vivo Effect of Angiotensin and Noradrenaline on the Proximal Tubular Reabsorption of Salt in Mammalian Kidneys  
*Acta physiol scand* 1965 64 167-175
- G Bojesen E and P P Leyssac The Kidney Cortex Slice Technique as a Model for Sodium Transport In Vivo A Qualitative Evaluation  
*Acta physiol scand* 1965 65 20-32
- H Bojesen E, P P Leyssac and B Svejgaard Nielsen Analysis of the Efflux Kinetics of Sodium from Small Cylinders of Rabbit Kidney Cortex  
*Acta physiol scand* 1965 65 103-110





# Table of contents

INTRODUCTION	11
CHAPTER I	13
A Early concepts of mechanisms in urine formation	13
a) Glomerular filtration – tubular reabsorption	13
b) Determination of GFR	13
c) Passive – active reabsorption	14
d) Tubular secretion	14
e) Tm limitation of rate of transfer of actively reabsorbed substances	15
B Basis of the accepted theory	16
a) Assumption I Clearance of inulin a measure of glomerular factors	16
b) Assumption II Proximal sodium reabsorption load dependent Distal sodium reabsorption Tm limited	17
c) Assumption III & IV Proximal sodium reabsorption normally gradient limited Urea contributes to establishment of the gradient	19
d) Assumption V The constancy of the hydrostatic pressure in the space of Bowman's capsule	23
CHAPTER II Trans cellular transport processes across the mammalian proximal tubule cell	III
A Rate limitation of active transport in general	28
B The rate limiting step in active transepithelial absorption	32
a) The rate limiting step of the sodium transport across amphibian skin – and urinary bladder	33
b) The rate limiting step of the mammalian proximal tubular reabsorption of salt and water	34
c) The rate limiting step of the mammalian proximal tubular reabsorption of glucose	50
CHAPTER III The problem	52
A Alternative models of load dependent mechanisms of proximal salt reabsorption	52
a) Gertz hypothesis	52
b) Helman's hypothesis	54
c) The general contemporary concept	55
B Investigations by indirect and direct methods by which to elucidate the problem	56
a) Indirect methods	56
b) Direct methods	61

CHAPTER IV	Consequences of $T_m$ limitation of proximal reabsorption of salt and water	69
A	The concept of the glomerular filtration rate	69
II	The tubular action of angiotensin	77
CHAPTER V		87
A	A regulated parameter	87
II	Conditions of changed release of angiotensin or renin	91
a)	Renal renin content in kidneys in which the release of renin and/or angiotensin assumably is increased	92
b)	Changes in lymph and plasma concentrations of renin and angiotensin	94
C	Hypotheses on the stimulus to angiotensin release and/or the function of the juxtaglomerular apparatus	97
CHAPTER VI	Some further observations relevant to the present hypothesis	111
a)	Hypothetical considerations on the location of the tubular action of angiotensin	111
b)	Glomerular filtration rate and distal salt load	112
c)	The dilution diuresis and expansion of the extracellular fluid volume	113
d)	The renal effect of noradrenaline and other pressor substances	115
e)	The importance of a voluminous proximal fluid reabsorption in mammalian kidneys	116
f)	The hypothesis considered in the light of evolution	117
FINAL REMARKS		120
APPENDIX	Statistical analysis	121
SUMMARY		125
DANSK RESUMÉ		131
ACKNOWLEDGEMENT		137
REFERENCES		139
SUBJECT INDEX		149

## Introduction

When introduced to renal physiology the student is impressed first by the wide aspects and possibilities opened with the development of direct methods in the study on water and solute transfer across the glomerular membrane and across the different segments of the tubular wall along the nephron. Since the time of the original micropuncture experiments on frog kidneys 40 years ago the microtechniques and analyses have been widely applied with increasing refinement and perfection to studies on transfer processes in single nephrons of the mammalian kidney especially during the last decade. Thus the renal physiologist has gained a rather perfect tool for investigations of the different role played by glomerular and tubular factors in changes observed in the excretion of water and solutes.

During recent years the direct methods have enlarged our understanding of the movements (reabsorption and/or secretion) of the individual constituents of the glomerular ultrafiltrate in the different segments of the whole nephron. These methods have provided also a certain information concerning the individual processes of the transcellular transfer viz whether they are ascribable to passive or active energy requiring forces.

The introduction of these direct methods has contributed greatly also to the establishment of our present theory of the renal mechanisms by which a concentrated and dilute urine in the mammalian kidney is elaborated. Consequently it has contributed also to our understanding of the osmoregulation of the extracellular fluid space. In this model the structural and functional correlations have been synthesized into a system which in a simple and ideal way seems suitable for a hormonal regulation of water excretion independent of simultaneous solute excretion.

Conversely, the renal handling of salt seems to be a problem which is becoming increasingly complicated even though innumerable data concerning the integrated renal handling of salt have been obtained by clearance studies on the renal salt excretion in mammals. Studies which have been carried out under a great variety of experimental conditions, normal and pathological states and under the influence of various hormones and other substances. Data obtained by direct methods from the different segments of the nephron have failed to support some indirect evidence obtained from clearance studies as will appear from the following analysis, many of the data are seemingly controversial or obscure. The link by which the functional states of the

proximal and distal tubular segments are correlated, no doubt a *sine qua non* for a regulation of the salt and water excretion, has been searched for although without success. Further, any synthesis of the accumulated knowledge into a model which is generally acceptable on the basis of our present knowledge and which describes in quantitative terms the renal mechanisms involved in the regulation of the salt excretion, and thereby of the volume of the extracellular space, has not been proposed. This fact may appear rather surprising since it had been possible at an earlier stage to describe in quantitative terms the process of transfer of another reabsorption substance, viz glucose and to bring into accordance the directly obtained knowledge concerning the function of the proximal tubular segment and the integrated tubular functions observed in clearance studies.

Hence it seems justified to question whether or not some essential feature in the renal handling of salt actually has been recognized as a problem worth investigation by a direct method. It is my intention in the present work to draw the attention primarily to one feature in the renal handling of salt which so far and with one single exception has not been fully recognized as a problem of major importance. The problem was clearly formulated by the question whether or not the rate of tubular transfer of the major part of filtered salt and water is dependent on the filtered load per se. In order to make the reader comprehend the origin of this problem the difficulties involved in the recognition and solution of the problem, and the inferences of a final answer to the question a brief and far from exhaustive review shall be given of the basis of the former generally accepted theory and the previous interpretations thereof.

In a subsequent discussion the problem thus raised is analysed on the basis of available data, the greatest importance being attached to data obtained by direct methods. Based on the conclusions drawn from this analysis and on basis of further experimental approaches to the problem of the regulatory mechanisms if any of the proximal tubular reabsorption of salt and water I shall attempt to bridge the gap between findings concerning the function of the single tubular segments in transfer of salt and water obtained by direct methods and the indirect evidence of the integrated function obtained from clearance studies. Finally it may turn out I hope that it will be possible on this basis to give an outline of some of the major features of the renal mechanisms involved in the regulation of the salt excretion and to correlate these functional concepts to macro- and ultrastructural equivalents.

It has been endeavoured first and foremost to restrict the present review exclusively to data of essential importance to the problem concerned rather than to give any exhaustive review and interpretation of the available data.

## CHAPTER I

### A EARLY CONCEPTS OF THE MECHANISMS IN URINE FORMATION

#### a) *Glomerular filtration – tubular reabsorption*

In the fundamental micropuncture experiments on frog kidneys Wearn and Richards (1924) directly established the two main principles outlined in Cushny's modern theory (1917), the processes of glomerular ultrafiltration and of tubular reabsorption. They demonstrated that fluid collected from the space of Bowman's capsule did not contain protein in measurable concentrations but contained chloride and glucose, while simultaneously collected final urine was almost free of these components in the filtrate.

#### b) *Determination of the glomerular filtration rate*

The requirements to a quantitative characterization of the tubular transfer processes of the separate components of the ultrafiltrate seemed fulfilled by the introduction of a quantitative method by which to estimate the rate of glomerular filtration of water (GFR) in the intact kidney by measurement of exogenous creatinine clearance (Rehberg 1926 a). By this indirect method it became possible to calculate the rate of reabsorption of a filtered substance as the difference between the rate of filtration and the rate of excretion of the substance studied.

It is outside the scope of this review to enter into a discussion about the objections later raised as regards the correctness of using the exogenous creatinine clearance as a measure of filtration rate, especially in human beings and apes. Only it shall be mentioned that Shannon and Smith (1935) demonstrated in normal human subjects that the clearance of inulin is independent of its concentration in plasma and somewhat lower than the creatinine clearance. Furthermore Shannon (1935 a) showed that the creatinine clearance was reduced to values approximately equal to values of inulin when the plasma concentration of creatinine was greatly increased and also during phlorizinization. In later clearance investigations inulin has become the standard substance of choice for measurements of the glomerular filtration rate.

Further insight into partial processes in urine formation was conditioned by the quantitative determinations of the filtration rate – single milestones on

the way to the generally accepted theory of the renal mechanisms of urine formation shall be briefly mentioned to form a basis of the following discussion

### c) *Passive - active reabsorption*

On the basis of clearance studies on the excretion of urea, Rehberg (1926) first emphasized the necessity of a distinction between passive back diffusion and active reabsorption (in the sense of reabsorption against a concentration gradient). He presented evidence of passive diffusion of urea from the filtrate to the plasma showing that the concentration index of urea always was lower than that of creatinine and that it decreased relatively with increasing concentration indices of creatinine and vice versa. Later investigations using direct methods have not yet been able to establish any active transfer of urea in the mammalian kidney.

Several investigators have found that the excretion fraction of glucose, water, sodium and other substances may vary more or less independently, and consequently Cushny's theory of the reabsorption of an ideal fluid of constant composition was completely abandoned, a theory which on the basis of similar arguments had been questioned even by Cushny himself. However, because of the low excretion fraction of these substances it goes without saying that the result only permits the conclusion that the theory of a constant composition does not apply to the total reabsorption. It was not excluded that some possibly a great fraction of the filtrate is reabsorbed as a perfected Lockes fluid.

### d) *Tubular secretion*

The participation of tubular secretion in urine formation previously suggested by several investigators on the basis of experiments with certain dyes as indigo carmine and phenol red was clearly demonstrated by Marshall (1930) in aglomerular kidneys of marine teleosts, the goosfish (*Lophius piscatorius*) and the toadfish (*Opsanus tau*). He observed that flow rate and composition did not differ essentially in urine obtained from glomerular as opposed to aglomerular marine teleosts. From both types of fish large amounts of magnesium sulphate phosphate creatinine, and undetermined nitrogen, and small amounts of urea were found to be characteristic components of the urine. Both types of fish can eliminate phenol red but a specificity in the elimination of certain foreign bodies and carbohydrates was observed. Only the glomerular kidneys were capable of eliminating ferrocyanide and glucose.

even when the concentration of glucose in plasma was high and phlorizin had been given

In clearance studies a tubular secretion of a substance is indicated only when its clearance is higher than the simultaneous clearance of inulin under steady state conditions. In this way tubular secretion of phenol red was demonstrated in dogs by Shannon (1935 b) who furthermore discovered that the clearance of phenol red decreased to values approaching or even below the simultaneous clearance of creatinine provided the plasma concentration of phenol red was increased above a certain level. This phenomenon (self depression), which has now been demonstrated for a large number of organic compounds was believed to be due to a saturation of some step in the transfer mechanism suggesting a maximal transfer capacity ( $T_m$  or  $T_{max}$ )

#### e) *$T_m$ limitation of rate of transfer of actively reabsorbed substances*

An apparently similar limitation in the rate of tubular reabsorption was demonstrated and clearly delineated in quantitative terms for glucose by Shannon and Fisher (1938). With increasing plasma concentration of glucose the quantity of glucose filtered would increase in direct proportion to the plasma concentration at a constant filtration rate but glucose did not appear in the urine until a certain critical plasma level had been reached the renal plasma threshold. Above this plasma level the quantity of glucose excreted per unit of time is also a linear function of the plasma concentration. The difference between the amount filtered and excreted per unit of time equal to the quantity reabsorbed per unit of time was found to be constant (maximal) over a great range of plasma concentrations and for prolonged periods of time. In other words the rate of reabsorption of glucose above the saturation level is independent of the filtered load (load is defined as (the volume  $\times$  the concentration) supplied to the cells per unit of time). Shannon considered this finding of a maximal reabsorptive capacity for glucose ( $T_{mG}$ ) due to the fact that each tubule cell is unable to transfer more than a certain (maximum) quantity of the substance per unit of time. In the following discussion the term  $T_m$  is used exclusively in accordance with this original definition which, of course does not exclude that the actual  $T_m$  value may be subject to hormonal regulation.

The concepts of urine formation thus delineated might be described in brief as a primary glomerular ultrafiltration followed by propulsion of the filtrate along the tubular lumina attributable only to a hydrostatic pressure head determined by the difference between the pressure in the space of



Bowman's capsule and the renal pelvic pressure. During the flow of filtrate along the tubular lumina a certain fraction of the filtered water and solutes is reabsorbed, either by passive back diffusion (e.g. urea and other waste products) or for other substances – the Tm substances – by transfer mechanisms of limited reabsorptive capacity. Still other filtered substances may not be reabsorbed at all, while some organic compounds may be excreted in the urine by mechanisms involving in part tubular secretion of limited rates of transfer.

## B BASIS OF THE ACCEPTED THEORY

### 1) *Assumption 1 Clearance of inulin a measure of glomerular factors*

Rehberg's estimation of glomerular filtration rates in clearance studies of 100–150 ml/min indicated that about 98–99 per cent of filtered salt and water is reabsorbed in the normal human kidney, an observation which later has been confirmed in almost any mammal investigated, in the physiological state the excretion of sodium chloride will never exceed 5–10 per cent of the filtered load. In accordance with the filtration reabsorption theory and on the basis of simple hydrodynamic considerations such low excretion fractions of salt and water would indicate that any interference with the tubular reabsorptive capacity instantaneously will reduce the rate of filtration, as realized clearly by Rehberg (1929). He writes: „Es ist doch ziemlich einleuchtend dass der nach der Filtration restierende Druck der also dazu ausreichen soll erst die totale Filtratmenge bis zu der eventuellen Resorptionsstelle und dann die viel kleinere übrigbleibende Harnmenge zur Blase zu treiben, nicht ausreichen kann um die totale Menge zur Blase zu treiben. Eine Lahmung der Rückresorption muss augenblicklich eine starke Herabsetzung der Filtration herbeiführen.“

It might have been relevant to ask now whether this also applied to such parallel changes in the rate of filtration and reabsorption of salt and water which occur physiologically, i.e. whether or not the spontaneous range of variations in the filtration rate (about 80–140 ml/min/1.73 m<sup>2</sup> surface area in man), which might occur without significant changes in the rate of excretion of salt and water, is determined by primary changes in the rate of reabsorption. But unfortunately this natural question was not asked. On the contrary it was generally accepted without criticism or discussion that the determination of the glomerular filtration rate is a pure measure of the functional state of the glomerular vessels and that occurring changes in the rate are brought about only on account of changes in the vascular resistances.

in the afferent and/or efferent arterioles. Actually the term glomerular filtration rate was apparently a conceptual trap. It shall be emphasized that this assumption, which till now has not been experimentally confirmed directly, formed the basis upon which Homer Smith built his theories on the renal handling of salt and water, as they are outlined in his book *The Kidney* (1951).

The clear logic in Rehbergs above quoted statement may have been neglected by later investigators or taken only as an indication of a gross functional glomerulo tubular balance, which most recently has been subject to a careful experimental investigation by direct methods. Potassium dichromate or mercuric chloride was administered to rats in order to provoke necrosis of different parts of the tubular segments. After stated periods of time blood sampling, urine collections, and micropunctures of proximal and distal tubules were performed by Gottschalk and his group (Biber Mülle and Gottschalk 1964). Clearances of inulin, tubular fluid to plasma ratios (F/P) and urine to plasma ratios (U/P) of osmolality, inulin, urea, and para amino hippurate (PAH) were determined. The tubular lesions and sites of punctures were analysed by microdissection and described by Oliver and MacDowell (1964). A good structural functional correlation was observed as regards extent and localization of the tubular necrosis and functional disturbance. On an average the inulin clearance (51 measurements) was reduced by two thirds in the dicromate animals, in which the tubular necrosis was approximately 60 per cent of the proximal convoluted segment in accordance with Rehbergs prediction. Furthermore the F/P ratio of PAH and urea in distal tubules were greatly reduced as compared to normal controls. When however these F/P ratios were divided by the F/P ratio of inulin from similarly located puncture sites the ratio was not significantly different from that observed in normal rats. Since the F/P ratio of inulin depends primarily upon the proximal reabsorption of sodium and water, and since necrosis of this segment will affect sodium and water reabsorption as well as the ability to secrete PAH, a normal ratio (F/P PAH divided by F/P inulin) in injured kidneys indicates merely that the reabsorption capacity and the ability of secretion is equally impaired, in accordance with expectations and the conclusions drawn.

b) *Assumption II Proximal sodium reabsorption load dependent  
Distal sodium reabsorption T<sub>m</sub> limited*

A very close parallelism between the filtered load and rate of reabsorption of salt and water which was established in numerous clearance studies in

the years between 1930-1910, suggested furthermore an intimately adjusted physiological glomerulo-tubular balance. This fact offered serious difficulties of comprehension and quantitative characterization of the transfer process involved according to the current concepts of the  $T_m$  limitation of the 'actively' reabsorbed threshold substances. Micropuncture experiments in rats by Walker et al (1941) demonstrated that by far the major fraction of the filtrate was reabsorbed proximally, as suggested by Rehberg (1926 a and b). By analyses on fluid collected from the proximal tubules these authors showed that the F/P ratio of creatinine had increased to a value of about 2.5 (2/3) halfway down the convoluted segment indicating by extrapolation that about 75-80 per cent of the filtered load of water had been reabsorbed at the end of the proximal convoluted segment. Furthermore the osmotic pressure of proximal fluid was found to be isosmotic with the plasma regardless of the site of puncture, indicating that a similar fraction of the osmotically dominant solutes (sodium, chloride and bicarbonate) had been reabsorbed at this site. These observations have been repeatedly confirmed by later investigators using the same direct method (e.g. Lissiter, Gottschalk and Mylle 1961, Windhager and Gebisch 1961 b). Altogether the findings indicated that changes in the distal reabsorption of salt and water will have but insignificant effect upon the total reabsorption of either constituent, as long as the fraction of salt and water reabsorbed proximally is large and remains fairly constant. The absolute amounts of both salt and water reabsorbed per unit of time will change in almost direct proportion to the filtration rate as long as the rate of excretion of salt is low as compared with the filtered load. Indeed the rates of filtration and reabsorption of salt are normally very nearly equal and hence the rate of reabsorption appears to vary in direct proportion to the filtered load as stated by Smith (1951, p. 312). The implication in this statement is that the filtered load of salt represents the independent variable parameter in accordance with the prevailing assumption. According to this assumption (that the determination of inulin clearance is a measure solely of the functional state of glomerular vessels) it might be possible theoretically to characterize the process of salt and water reabsorption quantitatively by the clearance method. In this case apparently the tubular reabsorption rate of salt would be an unanalysed function of the filtered load. The concept of a salt  $T_m$  was thereby invalidated and could hardly be maintained. If on the other hand the rate of filtration were determined by the rate of reabsorption of salt and water, as it might be if the above quoted statement by Rehberg (1929) were to apply also to normal conditions any characterization of the transfer process of salt and water by clearance measurements would of course be meaningless.

It was realized however that a distinction between glomerular and tubular

factors using the clearance method involved great difficulties with respect to salt and water, since the variability of the glomerular filtration rate during registration may be appreciable, successive measurements cannot be made in which variations between the individual measurements are less than 5-10 per cent indeed a very large variability as compared with the rate of salt excretion. Hence reabsorption rates of salt can only be evaluated under identical conditions of plasma sodium concentrations and filtration rates (i.e. of filtered loads) and relationships of excretion rate to load and capacity cannot be evaluated during, or immediately following a change in the load using the conventional clearance technique as emphasized in several reviews (e.g. Smith 1951, Wesson 1957, Kruhoffer 1960).

c) *Assumption III-IV Proximal sodium reabsorption normally gradient limited Urea contributes to establishment of the gradient*

A further and essential step to a seemingly acceptable theory of the mechanisms of renal tubular transfer of salt was presented by Wesson, Anslow and Smith (1948). These authors showed in dogs that as much as 60-65 per cent of the filtered water may be excreted in the urine during osmotic diuresis induced by infusion of mannitol solutions while at the same time only 15-25 per cent of the filtered sodium is excreted (indeed a considerably increased excretion fraction of sodium). In these experiments the flow of urine was very great and possible changes in the composition of the urine affected by the distal tubules might safely be considered relatively insignificant for which reason the composition of the final urine might be taken as an expression of the composition of the tubular urine at the end of the thin limb of Henle. This observation demonstrated that the reabsorption of sodium in the proximal tubules to some degree might be independent of the reabsorption of water and that sodium reabsorption in this segment is an active process which may proceed against a chemical (concentration) gradient. This finding was confirmed in similar experiments in rabbits by Kruhoffer (1950). Direct evidence from later micropuncture studies have fully established the active character of the proximal sodium transfer.

By the method of stop flow perfusion of single Necturus proximal tubules Solomon and his associates (Shupp et al. 1958) introduced into the lumen perfusion fluids containing different concentrations of sodium chloride and low concentrations of  $^{14}\text{C}$  labelled inulin. The fluids were made isosmotic to Necturus plasma by addition of mannitol. By analysis of the inulin concentration in the fluid collected at the end of the experiment from the lumen blocked by mineral oil on each side of the perfusion fluid the percentage of

water reabsorbed was calculated by the degree to which the inulin had become concentrated. They showed that fluid can be reabsorbed from a solution containing sodium chloride in concentrations even down to 65 meq/l into plasma containing 100 meq/l (Windhager et al 1959). With a lower salt concentration in the perfusion fluid water would move into the luminal fluid from the outside. These experiments indicate an active reabsorption of sodium or chloride, water following passively, if water were actively transported it might be difficult to understand why it could not be reabsorbed from solutions of low as well as high salt concentrations in which osmotic pressures were identical. These observations were confirmed in similar experiments in rats by Windhager and Giebisch (1961 a) in which a limiting concentration gradient of 10-60 meq/l was established within a few seconds. Similar findings during free flow conditions of sodium chloride reabsorption against a concentration gradient induced by mannitol infusions in rats were also reported by Windhager and Giebisch (1961 b), altogether indicating that sodium and/or chloride ions are actively transported.

The demonstration first by Solomon (1957) and by Giebisch (1958) of a transtubular electrical potential difference in proximal tubules of rat kidneys of 19-39 mV (on an average 20 mV), which was reversibly abolished by interruption of the renal blood circulation, indicated that sodium ions are actively transferred across the cellular wall. Measurements by Giebisch (1958) and Whittembury (1960) of an electrical asymmetry in the proximal tubular cells of *Necturus*, in which a potential difference (PD) of 74 mV across the peritubular membrane (cell negative) and a transtubular PD of 20 mV (lumen negative) was recorded, indicated a PD of 54 mV (cell negative) across the luminal membrane. This observation suggested a localization of the active step in the transfer process at the peritubular side of the cell. Finally Eigler (1961) using *Necturus* and Windhager and Giebisch (1961 a) using rats have demonstrated that short circuit currents obtained from single proximal tubules are approximately equivalent to net sodium reabsorption measured under similar experimental conditions. Thus in agreement with the conclusions drawn by Wesson, Anslow and Smith (1948), it seems safe to consider the proximal sodium reabsorption as an active process proceeding against a chemical and/or electrical gradient (uphill transport).

The increased excretion fraction of sodium to values well above 20 per cent found in the above cited experiments of osmotic diureses by Wesson, Anslow and Smith was assumed to be due to the flow rate (greater load) which had risen to a level above the capacity of the distal tubules. The observation in these latter experiments that a concentration gradient might have a rate limiting effect on the sodium reabsorption rate in the proximal tubules was

in striking contrast to the apparent lack of such gradient limitation of distal tubular sodium reabsorption since it was known that almost all sodium and chloride may be removed from the urine under conditions of salt depletion. Furthermore, experiments on volume expansion by rapid infusion of Locke's fluid (e.g. Wesson et al 1950) had shown that the rate of excretion of sodium had rapidly increased (within 20-30 min) parallel with increases in the glomerular filtration rate and renal plasma flow. Conversely studies on the effect of changing the renal arterial pressure showed that reduction in one of the kidneys of the local arterial pressure below 80-90 mm Hg was accompanied by reductions both in the rate of filtration and in the rate of excretion of sodium (Selkurt, Hall and Spencer 1949). This result was confirmed in subsequent publications by Blake et al (1950), Mueller et al (1951) and Selkurt (1951 a), who also observed that minor reductions in the renal arterial pressure on one side might reduce the sodium excretion rate on this side without affecting the rate of filtration to any detectable degree. These latter observations which shall be further discussed in a later chapter were apparently not considered to be significant and did not throw any serious doubt on the idea of a load-dependent sodium reabsorption.

In order to correlate the above and other observations into a satisfactory system the hypothesis was first proposed by Wesson, Anslow and Smith (1948) and later elaborated by Smith (1951) that the reabsorption of sodium takes place in two stages: (1) a *proximal process* involving approximately four fifths of the normal filtered load which process is a linear function of the load; (2) a *distal process* involving approximately one fifth of the filtered load which in contrast to the proximal tubular process is rate limited by a maximal transfer capacity ( $Tm^d_{Na}$ ).

The evaluation of the experiments underlying these hypothetical concepts are evidently open to criticism and severe scepticism since the clearance technique used in these investigations by no means was designed to follow a rapid sequence of events. Consequently the results obtained could not establish whether the observed changes in the rate of filtration were caused by possible primary changes in the rate of proximal reabsorption (changes of a capacity) or whether such changes in the transfer rate were events - automatically adjusted - secondary to changes in the filtered load. The same argument applies also to the usual interpretations of recent micropuncture data which are said to support the theories advocated by Smith. It was shown that the same fraction (75-80 per cent) of the filtered volume had been reabsorbed at the early part of the distal tubules under antidiuretic conditions (Lassiter, Gottschalk and Mylle 1961) as well as under conditions of isotonic or hypertonic salt loading (Giebisch, Klose and Windhager 1964) in spite of consider

able increases in the glomerular filtration rate under hypertonic loadings. Similarly, insignificant differences only were observed in this fraction of the filtered volume reabsorbed proximally in antidiuresis and diabetes insipidus, in spite of an about 50 per cent reduction in the filtration rate in the latter condition (Gertz, Kennedy and Ullrich 1964). Again these investigations do by no means permit the distinction between primary and secondary events, since the collections of luminal fluid were not completed nor initiated until several minutes (more than 15 min) after the load had changed. Therefore the results do not at all prove, nor do they even permit the suggestion, that the proximal reabsorption rate has changed as an automatical consequence of the load response.

In order to explain such postulated automatical adjustment of the proximal rate of sodium transfer, Smith and his collaborators inferred on the basis of the observation of a proximal rate limiting concentration gradient for sodium during osmotic diuresis that the reabsorption of sodium also *normally* occurs against a moderate concentration gradient (see Smith 1951, p. 311). It was further assumed that only a small fraction of filtered urea is reabsorbed proximally. Thereby the osmotic effect of urea might supply a basis for the establishment of the concentration gradient for sodium in addition to a relatively retarded diffusion of water. Thus increases in the filtered load would serve to diminish (or eliminate) the concentration differences by increasing the volume of fluid presented to the tubules. Thereby the absolute rate of sodium reabsorption would tend to increase *pari passu* with the filtration rate and *vice versa*. But naturally evidence obtained under conditions of mannitol diuresis does not allow any such inference to mechanisms operating under physiological conditions. Furthermore a few direct observations in rats on the sodium concentration in the proximal tubular fluid and in plasma (Walker et al. 1941) during moderate saline diuresis had already indicated that no such gradient was established — at least not half way down this segment. These direct observations have been confirmed in several later micropuncture studies in rats during saline diuresis and antidiuresis (e.g. Windhager and Giebisch 1961 b, Ullrich et al. 1963). It was shown unequivocally that the fluid to plasma ratio of sodium ( $F/P Na$ ) in the entire convoluted part of the proximal tubules remains as close to unity, as might be obtained with the analytical techniques applied. Also it was shown (Lassiter, Gottschalk and Mylle 1961) that about 50 per cent of filtered urea is reabsorbed at the end of the proximal convoluted segment (about 60 per cent of the total proximal length). In this context a more essential importance is attached to their demonstration that the proximal  $F/P$  urea which increases in the most early part of this segment (first 10–15

per cent) to an average of 15 shows no tendency to increase or to decrease further along the accessible part of the convolution, indicating that urea is reabsorbed along with sodium and water in the same proportion as in the tubular fluid. Hence the osmotic activity of urea cannot contribute to the establishment of a concentration gradient for sodium in this segment.

d) *Assumption V The constancy of the hydrostatic pressure in the space of Bowman's capsule*

A highly fundamental consequence or underlying assumption implied in the generally accepted concept of a load dependent proximal sodium reabsorption is the postulate that the hydrostatic pressure in the space of Bowman's capsule were a constant parameter equal to the interstitial pressure notwithstanding the great changes in the rate of filtration. The flow of fluid across the glomerular membrane and further along the tubular lumina until the renal pelvis must be brought about by a common pressure head. By Kruhoffer (1960) termed the glomerular propulsive pressure which is equal to the difference between (the hydrostatic pressure in the glomerular capillaries - the colloid osmotic pressure of the plasma proteins) and the renal pelvic pressure. A part of this pressure head the effective filtration pressure ( $\frac{1}{2}$  glomerular propulsive pressure - hydrostatic pressure in the space of Bowman's capsule) is used for the formation of the total filtered fluid volume. Apparently the general assumption that changes in the glomerular filtration rate are caused primarily and exclusively by glomerular factors (i.e. by changes in the glomerular propulsive pressure) implies that the capsular pressure must be constant.

Within the accuracy of the available technique any significant pressure decline along the proximal convoluted segment cannot be demonstrated (Gottschalk and Mylle 1956) and consequently the resistance to fluid flow along this segment must be very low. Under antidiuretic conditions the control pressure averaged in the proximal tubules 12.5 mm Hg and in the distal tubules 6.7 mm Hg (Gottschalk and Mylle 1957), indicating a considerable resistance across the loop of Henle. During osmotic diuresis the distal intratubular pressure increased to values almost identical to those measured in the proximal tubules under these conditions, indicating a further considerable resistance to flow at a more distal site, which might be rate limiting to flow at high urine flow rates. The ducts of Bellini were considered to be the site of this distally located resistance. Since the major fraction (75-80%) of the filtered volume is reabsorbed proximally, it seems evident that an increase in the glomerular propulsive pressure (accelerating the filtration rate although



not necessarily to measurable degrees) will be followed by an almost parallel increase in the proximal intratubular (viz capsular) pressure – i.e. a changed balance between tubular resistance and volume of fluid presented – unless either the resistance is reduced or the rate of proximal reabsorption is increased. According to the opinion of Smith such increase in the reabsorption rate would be an automatic secondary event as outlined above. If, on the other hand, the rate of proximal reabsorption were independent of the filtered load (i.e. a process of Tm character above the saturation level) any increase in the glomerular propulsive pressure would be followed by a similar change in the capsular pressure and no significant (measurable) change would occur in the effective filtration pressure (or glomerular filtration rate) until some other factor(s) change(s) the reabsorptive capacity (or tubular resistance).

Indirect evidence against the implication of a constancy in the capsular pressure following changes in the glomerular propulsive pressure was presented by Bojesen (1954 b). In order to measure correctly inulin clearance in dogs even under conditions of changing urine flow, corrections were applied to avoid errors originating in tubular delay (at low urine flow) and dead space errors by determinations of 'appearance time' of injected dye and determinations of renal pelvic volumes at different urine flow rates (Bojesen 1954 a). By this careful procedure the sequence of events in dogs was studied within the first 10–15 min following rapid infusions (200–300 ml per 20 kg body weight) either of colloid free Ringer solutions (dilution diuresis) or Ringer solutions containing high concentrations of dog plasma proteins. It was shown that changes in the oncotic pressure of the plasma (and thereby in the glomerular propulsive pressure) by 8 mm Hg failed to change significantly ( $\geq 10\%$ ) the rate of glomerular filtration within the first 10 min. Renal plasma flow (clearance of diodrast) remained also unaffected within this period of time. After 10–15 min the glomerular filtration rate and the renal plasma flow had increased to measurable degrees. On the assumption of constancy in the capsular hydrostatic pressure a change by 8 mm Hg in the glomerular propulsive pressure should change significantly the rate of filtration unless the pressure drop across the glomerular membrane were to exceed about 80 mm Hg which is incredible, or unless the resistance in the afferent and/or efferent arterioles had changed. The latter possibility seemed unlikely since the renal plasma flow remained unaffected. Therefore it was justified to conclude that because of a parallel change occurring in the capsular pressure the effective filtration pressure had not changed significantly within the first 10 min. This indirect evidence which is incompatible with the theories advocated by Smith, is confirmed by direct measurements of the

proximal intratubular pressure in rats under similar conditions (Gottschalk and Mylle 1956). These authors injected relatively similar amounts of dextrose solution (1-2 ml per 200-300 g of body weight) intravenously and observed by rapidly successive recordings of the intratubular hydrostatic pressure, an immediate pressure increase to an average of 11 mm Hg (5-25 mm Hg). The pressure then declined gradually to preinjection values in 4-19 min, averaging in 7.5 min. It is apparent from the figure that the mean systemic arterial pressure had not changed significantly at the peak of intratubular pressure increase (Gottschalk and Mylle 1956, fig. 2) and the authors found no correlation between arterial and intratubular pressure. Although not discussed in their paper, these direct observations agree remarkably well with Bojesen's results obtained by the modified clearance technique with respect to the absolute value of the pressure increase as well as the time interval between the lowering of the oncotic pressure and until significant changes in effective filtration pressure occurred.

Although it is not my intention here to enter into a profound discussion and provide an interpretation of the many phenomena linked to the concept of autoregulation of renal blood flow and filtration, two different types of response to the elevation of the renal arterial perfusion pressure may be of relevance in the present discussion since they may illustrate the significance of the intratubular pressure change to the effective filtration pressure, as indicated by the above observations. The phenomenon of an absence of changes or of moderate changes only in the renal blood flow and the glomerular filtration rate at changing renal perfusion pressures within the interval of 80 to 180 mm Hg has been extensively investigated under a variety of experimental conditions and by different techniques. Changes in the renal tissue pressure originally measured by the now almost abandoned needle technique, are measured by the wedged deep intrarenal venous pressure or by micropuncture of the proximal tubules and peritubular capillaries. These two methods give almost identical results as demonstrated by Gottschalk (1964 a). It has been observed repeatedly, especially by Hinshaw and his colleagues (see Hinshaw 1964) that the renal tissue pressure changes parallel with arterial perfusion pressure within the interval of autoregulation in the isolated kidney as suggested by Shipley and Study (1951). This finding is in contrast to observations from kidneys in situ by Thurau and Wober (1962) and Thurau (1964 a) where kidneys were claimed to be denervated surgically. In these in situ experiments high systemic blood pressures were obtained either by bilateral carotid occlusion or by procaine injection into both vagal nerves and the walls of the carotid sinus (neurogenic hypertension). It is not stated however whether the renal artery had been divided and later

reconnected (the only explicit way of denervation) or whether it had been stripped only. Any changes in the proximal intratubular or peritubular capillary pressures of kidneys *in situ* were not observed when the systemic pressure changed within the range of autoregulation. Whether solely myogenic or possible neurogenic or intrarenal humoral substances with vasoconstrictor and/or tubular effects are involved in these latter experimental conditions, the most reasonable general interpretation of the unchanged glomerular filtration rate seems to be that under conditions in which the tissue pressure increased in response to the elevation of the perfusion pressure the glomerular propulsive pressure as well as the intratubular pressure had increased in parallel, whereas neither the glomerular propulsive pressure nor the proximal intratubular pressure had changed significantly under the *in situ* conditions. Thus the effective filtration pressure has not changed significantly in any of these situations. As emphasized also by Thurau (1964 b) any detailed interpretation of the possible mechanisms involved in this phenomenon cannot be expected until aspects of the tubular reabsorptive function and hydrodynamic resistances to luminal flow are included in the discussion.

As apparent from the above discussion the current and conventional theory of the mechanisms involved in urine formation, especially those concerning the renal handling of salt and water is based upon a complex of interrelated assumptions. In these assumptions the absence of direct experimental evidence is conspicuous. Thus any evidence in support of the first assumption has never been provided (clearance of inulin a measure of glomerular vascular factors only), on the contrary gross disturbances in the tubular function suggest that this assumption may be untenable. The second assumption (load dependency of proximal sodium reabsorption) has not been and cannot be established by the clearance technique as usually performed since rapid sequence of events cannot be studied by this method. Conventional micro-puncture investigations have not been designed for this type of study either and data thus obtained have not been analysed with special reference to verify this assumption. Finally direct observations have excluded the validity of the assumptions III, IV and V. Therefore it seems rather pretentious when Homer Smith in his introduction to *The Kidney* (1951 p. XXII) states in an authoritative way: "Renal physiology has now passed into a quantitative phase where unsupported speculation and empirical description are no longer warranted. *It might rather be reasonable now to declare that there is no reason whatever for a maintenance without discussion of this theory.*" More approaches to direct experimental confirmation are highly desirable and as will be seen also available.

Before proceeding to the discussion of the experimental data which may disclose whether the major fraction of the filtered water and solutes is reabsorbed along the proximal tubular segment at a rate, which may be characterized as load dependent or load independent (i.e.  $T_m$  limited) it may be of advantage tentatively to have the process(es) of transcellular transport in the mammalian proximal tubules characterized at the cellular level. Such characterization, which now seems possible on the basis of a series of the most recent investigations using direct methods, may add to our present understanding or concept of the phenomenon of  $T_m$  limitation and gradient limitation of transcellular transports in the proximal tubules and thus will serve as foundation for the following discussion.

## CHAPTER II

# Transcellular transport processes across the mammalian proximal tubule cell

In the foregoing chapter no clear distinction has been made between the vague term transfer of filtrate and transfer of salt or sodium. The proximal reabsorption being an isosmotic reabsorption predominantly of sodium chloride, and bicarbonate ions and water and since it is known as discussed in the previous chapter (Ch I B c p 19-20), that sodium is actively reabsorbed against an electrical potential gradient the water following passively (osmotically), a preliminary quantitative description of transcellular fluid transfer therefore as a first approximation concerns primarily the uphill transport of sodium ions.

## A RATE LIMITATION OF ACTIVE TRANSPORT IN GENERAL

In attempts to characterize formally and experimentally an active transport process across biological membranes as opposed to diffusion a thermodynamic distinction has been preferred e.g. by Rosenberg (1948) and by Ussing (1949 c). Accordingly the term active transport whenever used in this review is limited to processes of transfer of chemical matter from a lower to a higher chemical or electrochemical potential (uphill transport), processes requiring energy generally by coupling to metabolic energy yielding processes of the cell. It should be emphasized that as a fundamental basis for the entire discussion of active transport phenomena the general thermodynamic functions are assumed to be well defined in the actual biological compartments or phases considered a postulate which well may be questioned, especially as regards systems far from a state of equilibrium.

The derivation from Fick's law of Ussing's flux ratio equation

$$(1) \quad \frac{M_1}{M} = \frac{C_1}{C}$$

has been of the greatest importance and of the utmost practical value for the design of the experimental approaches in this equation  $M_1$  is the

unidirectional flux in the direction (1→2) and  $M_0$  the inversely directed flux and  $C_1$  and  $C_2$  the concentrations of substance on the two sides of the membrane. In the case of charged particles (ions) a formally identical relationship was derived by Ussing (1949 b)

$$(2) \quad \frac{M_1}{M} = \frac{a_1}{a}$$

expressing that the flux ratio is equal to the ratio between the electrochemical activities of the ion on the two sides of the membrane when the ion moves passively in the uncombined state through a membrane where it is not subject to solvent drag. Thus in the case of insignificant net water transfer an active net transport of e.g. sodium ions is generally indicated by a flux ratio exceeding the one calculated from the equation (2). The usual procedure in the attempt to characterize active transcellular sodium transport is, by approximation, to consider the epithelial cell layer(s) as one single membrane, and further to define a quantity,  $E_{Na}$  which is denoted as the active transport potential of sodium or the driving force of the sodium pump, referring to an idealized system in which there is no electrical gradient nor any concentration gradient across the membrane. Such system may apparently be experimentally realized in a variety of epithelial tissues (e.g. the frog skin and the toad bladder), in which the normal electrical potential difference across the membrane is abolished by application of a short circuit current and in which identical sodium concentrations on the two sides of the membrane are established e.g. by bathing the tissue in Ringer solutions of identical ion composition on both sides. In this case we have

$$(3) \quad E_{Na} = \frac{RT}{F} \ln \left( \frac{M_1}{M} \right),$$

in which  $R$  denotes the gas constant,  $T$  the absolute temperature and  $F$  the Faraday number. The fluxes  $M_1$  and  $M_0$  may be determined directly by the double isotope tracer method. It is obvious that the rate of net sodium transfer through the membrane may be influenced by an opposed electrochemical potential possibly created by the active transport itself and will reach zero value when the electrochemical potential, against which sodium is forced, approaches the  $E_N$ . In this case the active transport of sodium itself creates a limiting potential gradient through the membrane and the net transfer rate may be characterized as gradient limited. The diuretic effect of e.g. mannitol loadings seems to be due predominantly to such an effect on the proximal reabsorption rate. The presence in the proximal fluid of the non reabsorbable but osmotically active mannitol will prevent water

from following the actively transferred sodium ions. The luminal fluid sodium concentrations are thereby reduced to values lower than those in the peritubular plasma, and the active transport now proceeds not only against the electrical potential of 20–25 mV but also against a chemical potential. As demonstrated by Windhager and Giebich (1961 a and b) the rate limiting sodium concentration difference in proximal tubules of rats was 40–60 meq/l. If the sodium concentration in the luminal fluid was reduced to establish a concentration difference exceeding this value the direction of net sodium flux was reversed.

As concluded by Ussing (1949 b) · Deviations from the flux ratio equation indicate that the ion does not diffuse in the free state only but in part at least as a component of some other moving particle in the membrane. The term carrier is usually applied to such other moving particles in the membrane with which the transported matter is coupled in complex formation. Although hypothetical in nature an extensive body of evidence now supports the carrier concept, which has provided a highly useful means by which to explain the qualitative and quantitative relations of several examples of transport in biological systems, as stated by Wilbrandt and Rosenberg (1961). The equation common to all types of carrier transfer relates the transfer rate ( $v$ ) to the movement of the carrier substrate complex in the membrane

$$(a) \quad v = D (CS_1 - CS)$$

in which  $D$  is a permeability constant (the diffusion coefficient of the transported matter in the particular membrane of a certain thickness), and  $CS_1$  and  $CS$  are the concentrations of carrier substrate complex on the two sides of the membrane respectively.

Quite generally the carrier system is characterized by the following steps. The binding between substrate and carrier, the diffusion of the complex formed and the splitting of the complex into substrate and carrier on the other side of the membrane. The kinetics of this transport of substrate will depend on the rate constants of these steps. In the most simple case in which the substrate ( $S$ ) is assumed to be in equilibrium with the carrier ( $D$  less than the rate constants of the reactions) and in which the further assumption is made that the diffusion constant of the free carrier is identical to that of the carrier substrate complex, the kinetics may be described by the equation

$$(b) \quad v = D C_t \left( \frac{S_1}{S_1 + K_m} - \frac{S}{S + K_m} \right)$$

in which  $C_t$  denotes the total concentration of carrier and  $K_m$  the dissociation constant of the carrier substrate complex (the Michaelis constant). It appears

from the equation (b) that the rate of transport will increase with increasing substrate concentration on one side of the membrane and since the last term the saturation term cannot exceed the numerical value of 1  $DC_i$  will indicate the theoretical maximum transport rate and may be substituted in the equation by  $V_{max}$

$$(c) \quad v = V_{max} \left( \frac{S_1}{S_1 + K_m} - \frac{S}{S + K_m} \right)$$

Thus  $DC$  or  $V_{max}$  is a capacity term of the carrier kinetics

According to the treatment of carrier kinetics in systems capable of uphill transport given by Wilbrandt and Rosenberg (1961) one of the possibilities of active carrier transport may be realized when the transport is coupled to the metabolic reactions which may change the  $K_m$  value to a different extent on the two sides of the membrane. In this case we have

$$(d) \quad v = V_{max} \left( \frac{S_1}{S_1 + K_1} - \frac{S}{S + K} \right)$$

If  $K \gg K_1$   $S$  will move uphill in the direction of (1→2) until a steady state is reached in which

$$\frac{K_1}{K} = \frac{S_1}{S}$$

Again we obtain an increasing transport rate with increasing substrate concentration on one side ( $S_1$ ) and in the non steady state in which no rate limiting potential gradient is created we may reach a maximum transport rate at the saturation level at which further increments in the substrate concentration no longer influences the rate. If this is the normal condition in the case of proximal sodium reabsorption the rate of sodium transfer may be characterized as *T<sub>m</sub> limited*

In the case of active transcellular net transport the situation obviously is far more complicated theoretically as well as experimentally since the transported matter has to move across at least two cell membranes which apparently must be quite differently characterized among other things as regards properties of permeability and capability of uphill transport. Hence a fairly detailed characterization of each of the bounding cell membranes and of the transport across these is necessary in order to explain quantitatively the overall net transfer. Besides when the discussion aims at an understanding at the cellular level of  $T_m$  limitation and gradient limitation of net transfer rates and a possible regulation of these rates it is equally necessary to be able to decide whether one or the other of the two cell boundaries constitutes the rate limiting step in the transcellular transport.



## B THE RATE LIMITING STEP IN ACTIVE TRANSEPITHELIAL ABSORPTION

Since active sodium transport across epithelial cells, which according to Ussing (1960) apparently is a characteristic of all animal cells which absorb sodium and chloride from the outside into the organism generally is considered to be due to essentially identical mechanisms in the tissues, in which it is established such as the frog skin the urinary bladder of the toad the intestinal epithelium of the toad and guinea pig and kidney tubules it may be appropriate briefly to draw into the discussion first some data and current hypotheses derived from observations on the most carefully studied tissues, the frog skin and the toad bladder. Thus, it was shown by Koefoed-Johnsen and Ussing (1958) that the electrical potential difference across surviving frog skins, which were treated either with copper ions in order to minimize the chloride permeability or were bathed in  $\text{Na SO}_4$  Ringer (sulphate being non-penetrating), was almost linearly related to the logarithm of the potassium concentration of the inside solution and with a nearly correct slope for a potassium selective electrode. Further it could be demonstrated that practically all exchange of potassium takes place between the cell interior and the inside solution, and that the inward facing membrane permeability of free sodium ions is very low. On the other hand, when the skins were copper treated or, even better, bathed in sulphate Ringers the potential increased almost linearly with the logarithm of the outside sodium concentration imitating the characteristics of a sodium selective electrode. It was concluded, therefore that the outward facing membrane in the absence of penetrating anions behaves as a sodium electrode [selectively permeable to sodium ( $-$  and lithium) ions] while the inward facing membrane behaves as a potassium electrode. Based on these observations the electrical potential difference across the living skin epithelium could be explained, if an active transport mechanism (a pump) were localized at the inner border which might force sodium ions from the cellular cytoplasm into the inside bathing medium and potassium from the inside medium into the cellular cytoplasm. The pump is believed to keep the intracellular sodium concentration low and fairly constant thereby creating an electrochemical potential gradient for diffusion of sodium from the outside solution into the cell, this would give rise to a sodium diffusion potential making the outside electrically negative to the cell. At the inner membrane potassium is assumed to move in the steady state in a closed circuit being actively transferred into the cell and diffusing from the cellular cytoplasm into the inside solution thereby giving rise to a second diffusion potential which makes the inside solution elec

trically positive in relation to the cell. In this way a specific active sodium transfer might be established and the electrical potential explained.

While the nature of the electrical potential still is intensively debated and probably may be of different types under different conditions and in different tissues attention should be drawn to two other essential features of the active transcellular sodium transport, implied in Ussing's model which seem to be generally accepted for all tissues investigated constituting a fundamental part of the hypothesis upon which all subsequent models proposed for each particular type of cells is based. 1) *The pump mechanism is assumed to be supplied with sodium ions from the intracellular cytoplasmatic sodium pool* and 2) *the intracellular sodium concentration is assumed to be maintained low by the same pump mechanism which effectuates the active transcellular net transport*.

a) *The rate limiting step of the sodium transport across amphibian skin and urinary bladder*

These latter assumptions which imply that sodium ions enter the cell passively down an electrochemical potential gradient may have some bearing upon the question of the rate limiting step of net sodium transport.

In the frog skin the rate of net sodium transport (outside→inside) was found to increase with increasing outside sodium concentration in the range from 1 to about 110 mmol NaCl although not in a linear proportion (Ussing 1949 a). With increasing sodium concentration the relative increase in net flux diminished in a way suggesting that the transfer asymptotically approaches a limiting maximum rate. Thus the transport process apparently shows saturation kinetics. The same type of kinetics was demonstrated by Frazier, Dempsey and Leaf (1962) in the case of sodium transfer across the toad bladder in which a transfer maximum, evaluated on the basis of the short circuit current was reached at an outside (mucosal side) concentration of about 60 mmol NaCl. Further their results indicated that the mucosal entry of sodium represented the limiting step of the rate of transfer since the tissue sodium pool, initially increasing with increasing sodium concentration in the mucosal medium also levelled off at a concentration of about 60 mmol NaCl as should be expected only if the mucosal entry of sodium was limiting the rate of net transfer. Altogether these findings indicate that sodium entry across the mucosal membrane although possibly passive involves some considerable interaction with the membrane or some constituent thereof (a carrier). Finally the stimulating effect of vasopressin on the active sodium transport (short circuit current) was also localized to the mucosal

membrane, as indicated by an invariable increase in the tissue sodium pool in the presence of vasopressin. Recently Sharp and Leaf (1963) reported that the tissue sodium pool of the toad bladder also had increased during aldosterone stimulation of the active sodium transport.

In the frog skin severe interference with the sodium pump, as indicated by a rapid drop in the electrical potential e.g. by ouabain treatment or a lowering of the pH of the inside bathing solution to 5.9, was not followed by a cellular swelling as might have been expected (MacRobbie and Ussing 1961), indicating a tightening both of the outward and inward facing membrane to ions. A low pH in the inside medium also reduced the water permeability of the inside membrane to about 50 per cent of the value at pH 8, and the authors conclude 'It may be that any interference with the pump mechanism is accompanied by a decrease in the passive permeabilities to ions and that the active ion transport and the passive fluxes are not entirely independent'.

If it were justified to generalize from these results obtained from some amphibian epithelia, which actually have far more resemblance to the mammalian distal tubular epithelium than to the proximal epithelium functionally as well as morphologically, it might *a priori* be expected or suggested that the rate of reabsorption of filtrate in the proximal tubules of the mammalian kidney (high outside (luminal) sodium concentration) were also limited by the transfer process at the luminal brush border membrane (morphologically the outward facing border).

b) *The rate limiting step of the mammalian proximal tubular reabsorption of salt and water*

No direct or unequivocal evidence has yet established whether the luminal or the peritubular membrane of the proximal tubule cell constitutes the rate limiting step of reabsorption. Some indirect evidence, apparently suggesting that the access of filtrate to the luminal brush border of the proximal cells might be of importance for the pump, was believed to support the contention that the luminal process of entry of ions to the cell is rate limiting (Leyssac 1965 a). It was observed in rats that as soon as the reabsorption of proximal luminal fluid was completed after interruption of the renal blood circulation and filtration a significant swelling of the proximal cells occurred. Since it was observed also that proximal cells of rat kidney cortical slices did not gain their original volume, but on the contrary swelled even more if transferred to oxygenated Ringer solutions or plasma, it was inferred that lack of oxygen probably could not be the only reason for the observed swelling (by

interference with the pump mechanism) and that the lack of luminal fluid (substrate) in part at least might be of importance for the swelling phenomenon. However, later investigations (unpublished) disclosed that the sodium concentration of similar cortex slices incubated in oxygenated Ringer solution depends on the incubating temperature and the efficiency of stirring. Tissue sodium concentration might be lowered by as much as 10–15 meq per kg of wet weight when slices were incubated either at 25°C as opposed to 38°C or under violent stirring with continuous supply of oxygen. The secondary swelling seen after incubation of slices in oxygenated Ringer without such stirring may therefore most likely be due to lack of oxygen; no valid arguments were provided which could possibly exclude that lack of oxygen might also be the only cause of the initial (or primary) swelling seen some 25 sec after interruption of the renal blood circulation. Thus the question may be regarded as completely unanswered.

A different approach to the problem may be an attempt to sum up data characterizing the individual transfer processes of each particular component of the reabsorbate across each of the two bordering membranes and evaluate these observations in relation to the rates of net flux. The prerequisite of the possibility of obtaining some insight into the characteristics of ion transports across the luminal and peritubular membrane has been the collection of data concerning the direction of net movement of the given ion species, estimates of respective ion concentrations on the two sides of the membrane and of the electrical potential profile of the proximal tubular cell. Direct measurements under free flow conditions and in the absence of osmotic diuretics of the electrical potential profile of the mammalian proximal cell not being available as far as I know, reference shall be made to that of proximal cells in *Necturi* in accordance with general practice (cf. Giebisch and Windhager 1964). This seems to be justified for the following reasons. First, Marsh, Ulrich and Rummrich (1963) have reported an electrical potential difference across the proximal peritubular membrane in rats of about 60 mV (cell negative) under microperfusion conditions of almost zero net fluid flux (cf. below). Whittembury (1964, 1965) found similar values across the peritubular membrane of guinea pig cortical cells in slices in which the tubular lumina are known to be completely occluded (Bojesen and Leyssac 1965) for which reason a net transport hardly can take place. Second, almost all essential features of the mammalian proximal reabsorption are similar in proximal tubules of *Necturi* kidneys. Thus as in mammals the proximal tubular reabsorbate is isosmotic with *Necturus* plasma (Windhager et al. 1959) the water reabsorption is passive and due almost entirely to the reabsorption of sodium chloride (cf. Ch. I p. 20). In both kidneys a transtubular electrical potential

difference of about 20 mV exists across the proximal tubular wall (lumen negative) (cf Ch I, p 20). Further, taking into account that the measurements were performed in different series of experiments using the same animal species, a fairly good agreement was obtained between the short circuit current in *Necturus* kidneys (corresponding to an active monovalent ion transport of  $4.6 \times 10^{-11}$  Eq/cm<sup>2</sup>sec Eigler 1961) and the net sodium flux of  $6.2 \times 10^{-11}$  Eq/cm sec as estimated from radioisotope measurements by the stopped flow microperfusion technique (Oken et al 1959). In the rat a similar close agreement between these two methods of estimating the active net sodium transport was obtained (the short circuit current corresponding to a net flux of  $3.4 \times 10^{-10}$  Eq/cm sec, as opposed to the net sodium flux of  $3.0 \times 10^{-10}$  Eq/cm sec) as shown by Windhager and Giebisch (1961 a). A peritubular potential difference of 70–74 mV (cell negative) in the proximal cells of mammalian tubules under physiological free flow conditions as measured directly in *Necturus* proximal cells e.g. by Whittembury (1960), therefore may be accepted as the most likely value. With a transtubular electrical potential difference of 20 mV (lumen negative) a potential difference of 50–54 mV (cell negative) across the luminal brush border is calculated.

The application of the microperfusion technique described by Gertz (1963) has been a particularly suitable approach to a characterization of ion transports across the two cell borders of mammalian proximal cells, by this technique a condition of zero net flux may be approached by the introduction in the single proximal lumen of a slowly penetrating non electrolyte such as raffinose. In brief, an isotonic aqueous raffinose solution is injected intraluminally between two oil columns. After an initial volume increase of the interposed aqueous solution due to influx of sodium potassium and chloride and accompanying water, the volume increase ceases again after about 50 sec to be followed by a slow and exponential volume decrease (Gertz 1963), during which the concentrations of sodium, potassium and chloride remain constant according to Kashgarian et al (1963). During this latter condition of exponential volume decrease the net fluid outflux is reduced to about 15–20 per cent of normal. It may be considered as an approximation to a steady state condition as regards the rapidly permeating ions. Under this experimental condition measurements of the transtubular electrical potential difference (between the isolated fluid column and the interstitium) of the concentrations of sodium potassium, and chloride in plasma and in the simultaneously recollected perfusion solution permitted an evaluation of the transtubular electrochemical potential differences of each ion species.

During the steady state condition with raffinose in the lumen the proximal transtubular electrical potential difference in rats amounted to an average of 24 mV (lumen negative), as opposed to 20 mV during free flow conditions. The mean sodium concentration was 149 meq/l in the plasma and 109 meq/l in the perfusion fluid (corresponding to 103 meq/l after correction to a state of absolutely zero net flux), giving a tubular fluid to plasma sodium concentration ratio ( $F/P_{Na}$ ) of 0.7 (Hashgarian et al 1963). From these data the active transcellular transport potential for sodium ( $E_{Na}$ ) was estimated from the equation given by Ussing (1960, p. 121)

$$E_{Na} = \frac{RT}{F} \ln \left( -\frac{c_i}{c_o} \right) + E + \frac{RT}{F} \ln \left( \frac{M_i}{M_o} \right)$$

(in which  $c$  and  $c_o$  denotes the peritubular and luminal concentrations respectively and  $M_i$  and  $M_o$  the unidirectional fluxes  $M_i$  indicating the direction of flux from the lumen to the peritubular interstitium). Since the net flux may be considered as approaching zero value the last term of the equation also becomes approximately zero. The proximal  $E_{Na}$  then was found to be 31 mV.

As mentioned above (p. 31) an electrical potential difference of 60 mV (cell negative) across the peritubular membrane was observed by Marsch Ullrich and Rummich (1963) under such conditions of approximately zero net fluid flux from which it follows that the luminal membrane potential is about 35 mV (cell negative). Since it is known also that the intracellular sodium concentration is considerably lower than that of proximal fluid and plasma (as confirmed by Bojesen and Leyssac 1965 cf. below) the active element of the transport process might be expected to be located at the peritubular end of the cell according to the general concept that sodium is pumped out from a homogeneous cytoplasmic pool. On the same assumptions it is inferred that sodium entry across the luminal brush border occurs passively down a considerable electrochemical potential gradient. But apparently sodium ions are not transported across this membrane by simple free diffusion as pointed out by Ullrich (Marsh Ullrich and Rummich 1963). If they were an electrochemical equilibrium across the luminal membrane should be approximated under the experimental steady state condition of approximately zero net flux. Quite obviously such equilibrium was very far from being approached. The intracellular sodium concentration in the equilibrium state at a luminal sodium concentration of 110 meq/l and an electrical potential difference of 35 mV (cell negative) would be of an order of magnitude of 500–600 meq/l. Such concentration of sodium in the cyto

plasmatic pool is clearly incompatible with the idea of isomolality of the tubular and intracellular fluids which seems generally accepted as well as with the general notion of a high intracellular potassium to sodium concentration ratio. The authors were therefore driven to conclude, that sodium ions were restricted from reaching equilibrium, a fact which might be explained by an active transport of sodium from the cell also into the proximal lumen. The opposite direction of net sodium flux into the cell was still believed to be due to free diffusion. It might be questioned whether or not the lack of equilibrium across the luminal membrane during the steady state conditions could be due to a low sodium permeability. This however does not seem very likely taking into account the extremely large net flux of sodium which normally takes place across the cellular wall as a result of still much larger fluxes in each direction.

Using the same method these investigators showed that potassium also is actively transferred across the proximal tubular cell. Under the stationary microperfusion condition a mean proximal F/P K of 0.91 was observed. With a transtubular electrical potential difference of 24 mV an active transtubular electrochemical transport potential ( $E_K$ ) of 26 mV was found. On the same general assumptions as in the case of sodium transport the high intracellular potassium concentration would necessarily indicate that potassium ions are actively translocated at least across the luminal membrane. The further movement of this ion species from the cell into the peritubular interstitium might then occur downhill by free diffusion and/or by some type of carrier mediated transport mechanism. As stated by the authors no data contradict even the possibility that potassium also is pumped actively into the cell on the peritubular side (morphologically the inside) as generally believed to be the case as e.g. in amphibian epithelia. Finally they proposed the hypothesis that an active linked sodium potassium exchange mechanism operates at all cell surfaces of the proximal tubular cell. They admit that such apparent transport symmetry obviously offers serious difficulties for a comprehension of the net orientation of this transport activity.

This problem seems even more complex when the chloride transport mechanism is included in the considerations. Thus although the overall proximal tubular chloride reabsorption apparently is of passive nature as the lumen is electrically negative in relation to the peritubular interstitium and as the luminal chloride concentration under free flow conditions can significantly exceed that of plasma a simple diffusion of chloride as the sole mode of transport probably is an inadequate explanation of the transfer mechanism which has been emphasized also by Giebisch and Windhager (1964). This statement is based on data presented by Kashgarian et al (1963),

obtained during the stationary microperfusion condition with raffinose in the luminal solution. In the steady state (the phase of exponential volume decrease) a mean F/P Cl of 0.97 was found (when plasma values were corrected for water content and Donnan effect). The observation of a trans-tubular electrical potential difference of 24 mV (lumen negative) under this condition approaching zero net flux permitted the conclusion that electrochemical equilibrium for chloride was not established but a force of 23 mV seemed to oppose the luminal entry of chloride into the cell which would correspond to an active transport of chloride from the cell into the lumen with an  $E_{Cl}$  of 23 mV (lumen negative).

In the case of urea a purely passive mode of reabsorption was suggested by Rehberg (1926 b) on the basis of clearance studies in man, as previously mentioned (Ch I, p 14). Although this postulate apparently has been supported by numerous similar studies in a great variety of mammals some doubt about this general statement has been raised, since it was observed that urea clearance in the kangaroo rat and the white rat might vary independently of the simultaneous inulin - or truly endogeneous creatinine clearance and that occasionally the urea clearance in the kangaroo rat might exceed that of inulin (B. Schmidt Nielsen 1955). Since a renal urea synthesis cannot be excluded and since it cannot be excluded either that the urea clearance might have exceeded the inulin clearance because of an excretion of urea deposited in a concentrated papillary interstitium at an earlier phase in addition to that filtered, no unequivocal demonstration of active transport of urea in the mammalian nephron has been presented yet neither with respect to secretion nor to absorption. It must be admitted, however as also emphasized by Gottschalk (1964 b), that the available data have not allowed any clear delineation of the character of urea movements in each of the nephron segments. In the proximal convoluted segment in which about 50-60 per cent of filtered urea is reabsorbed the tubular fluid concentration of urea invariably exceeds that of plasma on an average by a factor of 1.5 as shown directly in micropuncture studies (Lassiter, Gottschalk and Mylle 1961, Ullrich et al 1963). Thus if it may be assumed that the urea concentration in the cortical peritubular interstitial fluid is close to that in the peripheral plasma it seems safe to state that conditions apparently favour a passive overall transfer process at least in this segment of the nephron. The lack of direct knowledge of intracellular urea concentrations in the proximal tubular cell excludes an evaluation of the individual transfer mechanisms across the two bounding membranes.

Considered collectively these data present apparently a rather confusing complex of forces of various magnitudes favouring as well as opposing the net



movements of solutes and solvents from the proximal lumen into the peritubular interstitium. Actually each one of the new results seems to add to the confusion instead of completing a mosaic, in which a certain pattern was surmised. In our attempts to describe quantitatively the net transport of water and solutes across the proximal cells, in accordance also with the above mentioned observations, it is tempting to look for supplementary ideas, possibly obtainable by a comparison of the individual rates of net transport.

As referred to previously in this review (Ch I B c), it has been established by micropuncture studies that about 70–75 per cent of filtered water and sodium is reabsorbed at the end of the accessible part of the proximal convoluted (60–70 per cent of the total proximal tubule length) as indicated by an increase in the F/P inulin to a value of about 3 at this site with a maintained F/P sodium of unity. At this site 65–70 per cent of filtered chloride has also been reabsorbed. Wundtger and Giebisch (1961 b) measured tubular and plasma chloride concentrations in rats during infusion of a 0.9% sodium chloride solution and obtained proximal fluid to plasma ratios only slightly above unity (mean 1.03) independent of the distance from the glomerulus. Under antidiuretic free flow conditions a mean F/P Cl of 1.24 was found by Kashgarian et al. (1963), but also in this report it appears that this ratio neither increases nor decreases along the proximal convoluted. Thus chloride is reabsorbed along with sodium and water in the same proportion as in the tubular fluid. As mentioned previously (Ch I B c, p 23) this is also true for urea.

Recent results of proximal tubular fluid and plasma concentrations of potassium in antidiuretic rats have shown that the tubular fluid concentration is reduced to values below the plasma concentration before the fluid has reached 15–20 per cent of the proximal length and then maintains an unchanged F/p K of an average of 0.8 ( $0.79 \pm 0.03$  (SEM)) throughout the convoluted part (Marsh, Ullrich and Rumrich 1963). Similar values were reported by Malnic, Klose and Giebisch (1964) in control rats the ratio ranging from 0.69 to 0.91 in this report. In rats maintained on a low potassium diet this ratio was slightly higher, many values being close to unity (range 0.69 to 1.00). Also here the F/P K remained unchanged along the proximal convoluted in both groups of animals indicating that about 70 per cent of filtered potassium is also reabsorbed when the tubular fluid has reached about 65 per cent of the proximal length and that potassium ions are also reabsorbed together with sodium, chloride and water in the same proportion as in the proximal fluid. These recent investigations have confirmed in main the earlier report by Lichtfield and Bott (1962) in which the

F/P K was maintained close to unity along the proximal convolution in non diuretic rats

Finally it shall be mentioned that similar observations have been made with bicarbonate reabsorption in the proximal convoluted tubules of anti diuretic and water diuretic dogs (Clapp Watson and Berliner 1963) The mean bicarbonate concentration in the fluid collected from the proximal tubules was 16 to 17 meq/l in anti-diuresis and water diuresis respectively slightly less than the plasma concentration of 22 meq/l As in the case of sodium chloride potassium and urea the bicarbonate concentration showed no tendency to diminish (or increase) as fluid passed along this tubular segment either in anti diuresis or in water diuresis At the end of the accessible part about 70 per cent of filtered water, as inferred from the measured inulin ratios and about 75 per cent of the filtered bicarbonate had been reabsorbed The capacity of bicarbonate reabsorption, however seems to depend to a considerable degree on the experimental conditions and thus may present special and more complex problems than proximal reabsorption of sodium, potassium and chloride Rector, Bloomer and Seldin (1964) showed in normal anti diuretic rats that the proximal bicarbonate concentration decreased significantly along the proximal convoluted tubules as opposed to the above mentioned findings in dogs Although the degree of alkalosis in rats given  $\text{NaHCO}_3$  infusions and in hypokalaemic rats was comparable every sample of proximal fluid in rats receiving  $\text{NaHCO}_3$  had higher  $\text{HCO}_3$  concentration than plasma and the tubular fluid concentration was increasing along this segment whereas every proximal sample from hypokalaemic rats had a lower  $\text{HCO}_3$  concentration than plasma and it was decreasing along the convolution Any change in the proximal bicarbonate concentration could not be demonstrated however in samples collected from the same tubule (middle third of the convolution) before and after aortic constriction, which depressed the inulin clearance by 50-60 per cent On the assumption that the control clearance values have represented a certain part at least of the spontaneous range of variation it may be concluded that the proximal water and sodium reabsorption rate has been significantly depressed following the aortic constriction Thus the above observation that the tubular fluid bicarbonate concentration failed to change seems to indicate that the rate of bicarbonate reabsorption has been depressed to the same degree as the water and sodium reabsorption

It is apparent from these directly measured data that the rather stereotyped proximal reabsorption obviously has the main character of a bulk reabsorption of an aqueous solution of electrolytes and urea (of an ideal fluid) which

fact may seem rather peculiar considering the different characteristics of the individual processes and different forces acting on each one of the solute species at the luminal and peritubular membrane

The fact that variations in the rate of sodium and water reabsorption apparently are followed by parallel variations in the rate of chloride, potassium, bicarbonate, and urea reabsorption may seem even more peculiar. Imagining that e.g. the potassium reabsorption rate were constant and independent of the sodium and water reabsorption, physiological variations in the rate of reabsorption of the latter, by which the rate of filtration not only of sodium and water but also of the other constituents of the ultrafiltrate is changed in parallel, would change the fractional reabsorption of potassium (in relation to rate of filtration). Such induced difference in the fractional potassium reabsorption relative to that of water would be reflected in a change in the F/P concentration ratios at the end of the proximal convolution, which then might explain the observed scatter of these ratios. If however this were a valid explanation of this scatter, a significant increase in the scatter might be expected along the length of the proximal tubule. Such tendency is not established by the many reported data on potassium transfer, and in the case of bicarbonate the above mentioned evidence has suggested that the bicarbonate reabsorption rate does change in parallel with that of sodium and water. It therefore seems probable that *variations in the rate of proximal fluid reabsorption actually are variations in transfer rate of a volume of an aqueous solution of electrolytes and urea*.

This picture of the transcellular transport across the proximal tubular cell however, seems rather contradictory in many respects if considered from a quantitative point of view. Thus, the fairly close accordance between proximal net sodium flux as evaluated from the short circuit current and results obtained from micropfusion experiments makes it less likely that an active transport also of potassium and chloride participates significantly in the establishment of the total electrical current. The quantitative relationship might possibly be accounted for at the peritubular membrane of the cell, across which only sodium appears to be actively forced into the interstitial fluid with chloride and potassium ions moving downhill, and that the difficulties involved in the quantitative description arise at the luminal border. Furthermore other difficulties may be added.

Ullrich, Rumrich and Fuchs (1964) measured quantitatively the water permeability of the proximal tubular wall in rats using intraluminal infusions at constant rate of solutions containing sodium chloride at equilibrium concentration (the concentration at which net sodium chloride flux is zero in the presence of a non penetrating solute = 110 mmol NaCl) which had been made

hypo or hypertonic by addition either of raffinose or of mannitol. Aliquots of the infused solution were subsequently withdrawn at a more distal site of the convolution and the length of the perfused segment measured in the nephron, which later was isolated by microdissection. From the osmolalities of the recollected perfusion fluids and plasma samples, it was found that about 50 per cent osmotic equilibration was achieved after a flow along 200–400  $\mu$  of this segment (corresponding to a contact time of 0.3 to 0.6 sec), while complete equilibration was achieved at a perfused distance of about 1000  $\mu$ . The same values were obtained for water influx and outflux. When the data were plotted in a system with the logarithm of  $\frac{(100-A)}{10}$  as the ordinate ( $A$  denoting the per cent osmotic equilibration) versus the length of the perfused segment as abscissa a linear relationship was found, the slope of which is a relative measure of the water permeability. On the basis of this slope the luminal radius (used for calculation of the perfused area), the rate of infusion and the difference between the osmolality of the perfusion solution and plasma the coefficient of water permeability was calculated and found to be  $174 \times 10^{-8} \text{ cm}^3 \text{ cm}^{-1} \text{ sec}^{-1} \text{ cm H}_2\text{O}$  in the proximal convolution. Based on the known rate of proximal fluid reabsorption during free flow and this permeability coefficient it was calculated that the proximal fluid osmolality should be lower by 23 mosm than that of plasma if the actual reabsorption of water were to be accounted for on the assumption that the water flux were due to an osmotic gradient created by active salt reabsorption as generally believed. Since such difference in the osmotic pressure across the proximal tubular wall does not exist under free flow conditions their conclusion viz. that the water permeability actually is too low to account for the reabsorption of water seems justified. Consequently the transcellular water movement was postulated to occur, at least in part on account of hydrodynamic flow in subcellular compartments (channels). The space between the infoldings of the peritubular plasma membrane (the  $\beta$  cytomembrane) was believed to constitute the morphological equivalent of these micro channels.

Finally attention shall be drawn to the estimation of the proximal intracellular cation concentrations as calculated by Bojesen and Leyssac (1965) on the basis of analyses of sodium and potassium content in weighed fragments of rat cortical tissue frozen at the moment of removal. On the basis of the measured plasma space and the calculated proximal intraluminal volume the total content of extracellular sodium and potassium could be calculated and when subtracted from the measured total content in the tissue the intracellular sodium and potassium content could be calculated as the difference

The intracellular concentrations were found to be 5–15 meq  $\text{Na}^+/\text{kg}$  of cellular water and 125–135 meq  $\text{K}^+/\text{kg}$  of cellular water. This result may be compared with the one derived from an indirect approach by which the efflux curves of labelled sodium ( $^{22}\text{Na}$ ) from incubated rat cortical cylinders were subjected to graphical as well as numerical analysis, based on two different two compartment models, a non communicating and the more realistic communicating two compartment model respectively (Bojesen, Leyssac and Svejgaard Nielsen 1965). A fairly close agreement between the results of both types of analysis was obtained and a remarkably good fit with directly recorded experimental data was obtained by the numerical analysis. From the latter it appeared that about 80–85 per cent of exchangeable tissue sodium was in diffusion equilibrium with the incubating medium, the calculated virtual diffusion coefficient being  $7.3 \times 10^{-8} \text{ cm}^2 \text{ sec}^{-1}$  ( $25^\circ\text{C}$ ). From the separately measured concentration of tissue sodium in similar cylinders relative to that of the incubation medium it was calculated that the remaining 15–20 per cent fraction of exchangeable sodium, which was not in diffusion equilibrium with the medium, occupied a fraction of the total tissue water of 30–45 per cent. The sodium concentration in this compartment, representing predominantly the proximal intracellular space of the isolated tissue, was found to range between 35 to 55 meq/l which is considerably higher than the concentration calculated on the basis of analyses of cortical tissue frozen *in vivo*. It seems most likely, however, that the intracellular sodium concentration in cortical tissue incubated *in vitro* is somewhat higher than in the *in vivo* state. This statement is in agreement with the finding by Whittembury (1961, 1965) that the electrical potential difference across the peritubular membrane in cortical slices of a mammalian kidney was about 10–30 mV lower than values obtained from kidneys *in situ* (cf p 35) and also with the observation by Leyssac (1965a) that the proximal cells were swollen some 25–30 sec after removal of the kidney, at which time the proximal lumen are occluded. Based on these arguments it seems fairly safe to state that the intracellular sodium concentration of reabsorbing proximal cells *in situ* is very low, at least lower than 30 meq/l. This conclusion permitted a quantitative evaluation of the general assumption that transcellular transferred sodium ions are pumped actively from a homogeneous cytoplasmatic sodium pool across the peritubular membrane (Bojesen and Leyssac 1965). On the basis of a cellular sodium concentration of 14 meq/l, a plasma sodium concentration of 140 meq/l, and an electrical potential difference of 74 mV (cell negative) it was calculated that the minimum thermodynamic work required to accomplish the net transport of one equivalent of sodium would be 3200 cal at  $37^\circ\text{C}$  disregarding the (unknown) extra energy consumption involved.

in order to overcome the internal resistance to sodium movement. With a value of 25000-28000 cal corresponding to one equivalent of oxygen and 7-6 equivalents of sodium transported for each suprabasal equivalent of oxygen consumed in the mammalian kidney as found by several investigators (e.g. Deetjen and Kramer 1960, Lassen, Munch and Thaysen 1961), the efficiency will become 75-90 per cent. Since furthermore the resistance has to be overcome and some additional entropy production must be accounted for when sodium ions are transported to a higher electrochemical potential at the peritubular side of the membrane *it seems fairly safe to conclude that the general assumption underlying the calculation is open to serious doubt.* Hence an alternative assumption seems to be desirable as in the case of water movement. It has been suggested that transcellularly transferred sodium is not mixed with the cytoplasmatic sodium pool, but is confined to intracellular structures allowing a localized high intracellular sodium concentration (Bojesen and Leyssac 1965).

*In summary*, all these results have converged to the concept of a cytophysiological mechanism in many respects similar to micropinocytosis being involved in the transport of water and solutes across the proximal tubular cell, most likely being the physiological mechanism by which the tubular fluid with its dissolved constituents enter the cell from the tubular lumen. This hypothetical concept seems to solve at least most of the quantitative problems which have accumulated *passu* with some data recently obtained by direct measurements. These results, however, do not prove that a luminal transport process is rate limiting to the proximal net transfer of water and electrolytes (and urea) nor do they indicate that this process exhibits a  $T_m$  limitation although this possibility certainly should be seriously considered and experimentally investigated. All of the results discussed in this review are apparently compatible with such hypothesis, some even strongly support it. Furthermore electron micrographs of mammalian proximal tubular cells fixed in the reabsorbing state of function prior to the luminal occlusion and cellular swelling have disclosed the existence of an axial structure in each microvillus of the luminal brush border which morphologically is resembling a channel of about 10  $\mu$  (100 Å) in diameter (Hansen and Herman 1962). When fixed in this state of function the brush border is separated only by a rather narrow apical cytoplasmatic layer from the infoldings of the peritubular plasma membrane as seen from the electron micrographs presented by Maunsbach, Madden and Latta (1962). Several microvesicles are found in this apical cytoplasm. This finding further supports the hypothesis of a luminal micropinocytosis, the central channels in microvilli and the microvesicles being the morphological equivalent of this physiological transport mechanism.

Thus, according to the hypothesis proposed above, reabsorbed water and electrolytes (and urea) enter the proximal cells by a luminal micropinocytotic process and are moved in this way through the cell to its basal boundary, the infoldings of the plasma membrane or the basement membrane. Across this latter boundary membrane the further transport is achieved by an active, energy requiring mechanism, probably a sodium pump of a type so far undetermined i.e. the reabsorption has the character of a two step transcellular transport process. It should be emphasized that this theory implies that the active element under physiological conditions is supplied with sodium at a high concentration equal to the concentration in the proximal luminal fluid and furthermore that the transcellular transport mechanism in this specialized cell type apparently is spatially separated from the active mechanism by which the cell – like all other cells in the organism – maintains its cytoplasmatic cation concentrations. Naturally this does not exclude that these two active sodium transporting mechanisms may be accomplished by the same type of enzyme system such as an adenosin triphosphatase system as the one described by Skou (1957, 1964). In the presence of  $Mg^{++}$ , the activity of this enzyme system is characterized by its dependence on sodium and potassium ions. In the presence of potassium the enzyme activity increases with increasing sodium concentration, a plot of the effect of sodium concentration on the activity yielding a typical S shaped curve (Skou 1957). However, the maximum activity may be depressed by increasing the potassium concentration. In the enzyme system isolated from crab nerves a maximum activity was obtained in the presence of 100 mM or more of sodium and 20 mM of potassium with the steep part of the curve in the range of 10–50 mM sodium. Enzyme systems with similar main characteristics, located in subcellular particles have been isolated also from a large number of mammalian tissues including kidneys (e.g. Skou 1962) in which the affinities to sodium and potassium appear to be somewhat higher than in those prepared from crab nerve. On the main assumption that this enzyme system forms part of the transport mechanism (an active linked  $Na^+-K^+$  transport), by which the mammalian cell maintains a high potassium low sodium concentration, it is believed that the steady state is maintained at the steep part of the curve relating enzyme activity to sodium concentration. Thus if the intracellular sodium concentration tends to increase, the enzyme activity increases and consequently the active transport of sodium out of the cell increases. Were this hypothesis to be confirmed eventually it still might remain compatible with an assumption of a similar system forming part of the active element in the transcellular transport mechanism. If so and in accordance with the present hypothesis the enzyme system would operate under conditions of high sodium

low potassium concentrations (i.e. the concentrations in the micropinocytotic vesicles), at which the enzyme activity is at its maximum and uninfluenced by minor changes in the cation concentrations. If the active element of the transport process actually were to operate under optimal conditions as in such hypothetical case (and in the absence of a limiting electrochemical gradient) the rate of net transport might well be limited and regulated by the rate of the micropinocytotic step.

However, irrespective of the possible mechanisms of the active step in the over all process both steps in the transcellular transport may be rate limiting to the reabsorption, depending on the experimental situation. If conditions are established favouring the achievement of a sufficiently steep electrochemical gradient the basal thermodynamically active step may become rate limiting to the process. Then the rate of transfer is gradient limited as e.g. in mannitol diuresis during which the luminal sodium concentration becomes reduced. The active peritubular mechanism is then supplied with a low concentration of sodium and a limiting electrochemical gradient may be created across the peritubular membrane. Under other conditions in which such gradient is not established as under physiological conditions the luminal micropinocytosis may be the probable rate limiting step. That the reabsorption process under these latter conditions acquires the character of a Tm limited (or capacity limited) process independent of the filtered load will be comprehensible. The rate of micropinocytosis then determines the transport capacity and the kinetics of transport may be described in terms of carrier kinetics in which the luminal membrane with its adsorbed and/or enclosed solutes and solvent represents the carrier substrate complex proper (the moving particle of the cellular wall representing the membrane) (cf p 30-31). The extent to which the likeness with carrier kinetics is strictly tenable might reasonably be the subject of some further discussion.

For the sake of simplification the discussion may be restricted to the proximal sodium transport. Naturally the fact that changes in the filtered fluid volume (or amount of substrate) are without any influence per se on the transfer rate is not a problem. The problem arises when the sodium concentration is experimentally elevated e.g. by hypertonic saline infusion.

Two extremes among the possibilities may be considered.

*Possibility 1* The first possibility is that reabsorbed solutes are initially adsorbed (specifically) to the brush border membrane which subsequently by membrane flow carries the solutes into the cell by the infoldings (channel formation) at the tip of the microvilli. At a certain membrane flow rate the rate of solute transfer (or sodium transfer in this particular case) will be determined by the number of adsorption sites when amounts of solute are



sufficient to saturate the membrane. The accompanying water might be confined to the interspace between the membrane surfaces and in the microvesicles, in which the solute concentration is rather insignificant. This being the case an increased luminal sodium concentration ( $>140$  meq/l) would not influence the rate of transfer (assuming unchanged membrane flow rate), since the membrane (or 'carrier') was already saturated at the lower concentration. Thus the likeness with carrier kinetics seems perfect. Above the saturation level the rate of transfer will be maximal, determined only by the capacity term – the rate of membrane flow and microchannel formation –, which indeed may be variable. In this case it may be a matter of a true sodium  $T_m$  as well as of a volume  $T_m$ .

*Possibility II* might be that reabsorbed solutes and water were unspecifically ingested by the microchannel and vesicle formation without 'adsorption' to the membrane surfaces. In this case the rate of transfer would also depend on the rate of 'micropinocytosis', or volume transport capacity and it would be justified to use the term 'volume  $T_m$ '. But the rate of sodium transfer would not be strictly load independent – at least not at minor variations of the sodium concentration, on the contrary it would increase with increasing concentration. The kinetics of sodium transport would deviate from saturation kinetics at or above the saturation level and it would not be strictly correct to use the term 'sodium  $T_m$ '.

Finally all the (more likely?) possibilities between these two extremes should be considered which in principle correspond to the second case with respect to 'sodium  $T_m$ '. Chapman, Andresen and Holter (1964) have demonstrated in amoebae such a process which essentially is similar to the latter possibilities (those between the two extremes). They showed that labelled protein (albumin) could be ingested specifically by pinocytosis due to adsorption to the surface membrane. By immersion of amoebae in a solution containing  $^{125}\text{I}$  labelled albumin and  $^{14}\text{C}$  labelled glucose under conditions of low temperature, at which pinocytosis does not occur, a comparison of the ratios between the two compounds in the immersion solution and adherent to the amoebae showed that protein was absorbed preferentially by the amoebae with an enrichment factor of 36. By the same procedure, carried out at room temperature at which pinocytosis is induced by the protein containing medium and comparison of the activity ratios between the solution and within the amoebae, protein was found to have concentrated in the pinocytotic vesicle (or vacuole) with an 'enrichment factor' of 14. This observation allowed the conclusion that large amounts of protein, but insignificant amounts of glucose are first adsorbed to the cell while the fluid phase ingested consists of the bathing solution of protein and glucose.

These considerations may have some bearing on the interpretation of certain data obtained in clearance and micropuncture studies. It was observed in clearance studies on rabbits (Kruhoffer 1950, table VII and VIII) during hypertonic sodium chloride infusions and increasing plasma sodium concentration that the rate of filtration (inulin clearance) as well as the rate of sodium reabsorption increased initially. At high plasma sodium concentrations a constant inulin clearance could be obtained within a certain range of increasing sodium concentrations. By calculation of the amount of sodium (meq Na) reabsorbed per litre of filtrate formed it was observed that at a certain point the increase in sodium reabsorption rate ceases and at further increments in the plasma sodium concentration ( $\geq 180$  meq/l), the rate of sodium reabsorption decreases with increasing sodium concentration at a constant rate of filtrate formation. At these very high sodium levels there was a tendency to a reduction in the inulin clearance which fact was also observed in rats at plasma sodium concentrations exceeding 176 meq/l as reported by Giebisch, Klose and Windhager (1964) on the basis of a similar investigation. According to *possibility I* the initial increase in filtration rate and sodium reabsorption rate is due to an increased transport capacity (rate of micropinocytosis). At a certain point the latter has reached a maximum (zero inhibition). With increasing plasma concentrations the rate of sodium (and volume) reabsorption remains constant (saturation) all other factors being equal. The extra amount of sodium filtered is then excreted. With further increments of the luminal sodium concentration the rate of sodium reabsorption decreased corresponding to a decreased micropinocytotic activity (reduced capacity). In the case considered (*I*) also the volume reabsorbed per min would be reduced. It appears actually from the data given by Kruhoffer that the volume of fluid reabsorbed per min was reduced at the very high plasma sodium concentrations. It is therefore necessary to postulate that the high sodium concentration (or osmolality) has an inhibitory effect on the luminal activity of micropinocytosis.

If interpreted according to *possibility II* the initial rise in rates of filtration and sodium reabsorption is also due to an increased capacity of volume transfer which proceeds until a certain maximum is reached. At further increasing luminal sodium concentration a constant rate of sodium reabsorption would indicate that the rate of micropinocytotic channel and vesicle formation (capacity) is reduced along with increased concentrations in the ingested fluid. Also in this case the elevated sodium concentration must be assumed to have an inhibitory effect on the luminal process of volume transfer. Thus an apparent saturation or sodium  $T_m$  is reached at this state of function. Further elevations of the sodium concentration and inhibition of the micro

pinocytotic' activity may be followed by a decreased reabsorption rate of sodium, giving the appearance of a reduced sodium  $T_m$  ('substrate inhibition'). As far as I know no data are available which permit a clear distinction between these two possibilities of interpretation.

The most reasonable conclusion to be drawn from the above discussion seems to be that the data forming the basis of the present hypothesis of the mechanism of transcellular transport in the mammalian proximal tubules makes it permissible that the proximal reabsorption rate of salt and water be characterized as  $T_m$  limited.

c) *The rate limiting step of the mammalian proximal tubular reabsorption of glucose*

The question now arises to which extent the characteristics of glucose reabsorption in mammalian nephrons may be comprehended and adapted to the above hypothesis. The lack of glucose in the final urine at normal plasma concentrations and its presence in the glomerular filtrate in the same concentrations as in plasma clearly indicates that glucose is actively reabsorbed against a concentration gradient. It is indicated also in clearance studies (cf p 15) that at increasing plasma concentrations a certain glucose concentration level may be reached above which the rate of glucose reabsorption remains unchanged independent of the filtered load i.e. that a  $T_{mG}$  has been reached. Furthermore it has been established beyond doubt in several clearance investigations in mammals (e.g. Krühoffer 1950) that the glucose  $T_m$  remains uninfluenced by induced variations in the rate of filtrate formation above a certain level (10 ml/min in rabbits) and thus in the rate of reabsorption of salt and water. In this case salt and water serve as substances for reference. Apparently no special problems of interpretation have been attached previously to this fact. Above the glucose  $T_m$  level a hypothetical carrier was supposed to be saturated. In the view of the above hypothesis it may seem as a paradox that the understanding of the  $T_m$  limitation of glucose transfer rate represents a problem because according to this hypothesis a  $T_m$  limitation of the previous substances for reference (salt and water) may be described as a representative of luminal micropinocytotic activity. Thus if the rate of salt and water reabsorption is limited by the micropinocytotic activity under physiological conditions it may apparently be difficult to understand that the rate of net transfer of glucose remains uninfluenced by the rate of micropinocytosis. Theoretically, however the difficulties might appear to be less severe. The explanation is merely that different steps in the transfer process are rate limiting to salt and glucose

reabsorption the transfer step across the peritubular membrane being rate limiting to glucose reabsorption. No data are available by which it may be disclosed or suggested whether or not the limiting step to proximal net transfer of glucose actually is located to the basal (or peritubular) side of the cell. A more detailed discussion on the glucose transport in mammalian nephrons must be postponed until more direct evidence has been obtained together with a detailed physico-chemical characterization of the transcellular transfer mechanism(s) involved.

The data reviewed in this chapter which have thrown considerable doubt on some of the previously and generally accepted concepts and paved the way for an alternative hypothesis thus challenge future research work on the mechanism(s) of transcellular transport to provide new approaches including biochemical as well as cytophysiological investigations.

pinocytotic activity may be followed by a decreased reabsorption rate of sodium, giving the appearance of a reduced 'sodium  $T_m$ ' (substrate inhibition). As far as I know no data are available which permit a clear distinction between these two possibilities of interpretation.

The most reasonable conclusion to be drawn from the above discussion seems to be that *the data forming the basis of the present hypothesis of the mechanism of transcellular transport in the mammalian proximal tubules makes it permissible that the proximal reabsorption rate of salt and water be characterized as  $T_m$  limited*

c) *The rate limiting step of the mammalian proximal tubular reabsorption of glucose*

The question now arises to which extent the characteristics of glucose reabsorption in mammalian nephrons may be comprehended and adapted to the above hypothesis. The lack of glucose in the final urine at normal plasma concentrations and its presence in the glomerular filtrate in the same concentrations as in plasma clearly indicates that glucose is actively reabsorbed against a concentration gradient. It is indicated also in clearance studies (cf p 15) that at increasing plasma concentrations a certain glucose concentration level may be reached above which the rate of glucose reabsorption remains unchanged independent of the filtered load, i.e. that a  $T_m$  has been reached. Furthermore it has been established beyond doubt in several clearance investigations in mammals (e.g. Kruhoffer 1950) that the glucose  $T_m$  remains uninfluenced by induced variations in the rate of filtrate formation above a certain level (10 ml/min in rabbits) and thus in the rate of reabsorption of salt and water. In this case salt and water serve as substances for reference. Apparently no special problems of interpretation have been attached previously to this fact. Above the glucose  $T_m$  level a hypothetical carrier was supposed to be saturated. In the view of the above hypothesis it may seem as a paradox that the understanding of the  $T_m$  limitation of glucose transfer rate represents a problem because according to this hypothesis a  $T_m$  limitation of the previous substances for reference (salt and water) may be described as a representative of luminal micropinocytotic activity. Thus if the rate of salt and water reabsorption is limited by the micropinocytotic activity under physiological conditions it may apparently be difficult to understand that the rate of net transfer of glucose remains uninfluenced by the rate of micropinocytosis. Theoretically however the difficulties might appear to be less severe. The explanation is merely that different steps in the transfer process are rate limiting to salt and glucose

about 10 sec and the luminal diameter on an average  $31\mu$  corresponding to a net flux of water of  $5.5 \times 10^{-4} \text{ mm}^3 \text{ mm}^{-1} \text{ sec}^{-1}$ . From the flux of water the reduction of flow rate in the lumen at various distances ( $s$ ) from the glomerulus was calculated from the equation

$$\frac{V}{V_0} = 1 - \frac{2\pi r \bar{Q}_w s}{V_0}$$

in which  $V_0$  denotes the flow rate at a distance ( $s$ ) = 0 from the glomerulus (= rate of filtration from a single nephron),  $V$  the flow rate at a distance of ( $s$ ) and ( $r$ ) the luminal radius. The equation will be seen to imply that the flux term is constant at various distances from the glomerulus in agreement with Gertz' observation that the flux did not vary by more than 10 per cent along the accessible part (60–70 per cent) of the proximal segment. Inserting in the equation the obtained value of  $\bar{Q}_w$  (measured at a radius of  $15.5\mu$ ), a radius of  $10\mu$  and a value of  $V_0 = 4 \times 10^{-4} \text{ mm}^3/\text{sec}$  (calculated by Gottschalk and Mylle (1956) in rats under similar experimental conditions) it was found that the relative fluid volume reduction at a certain distance from the glomerulus agreed fairly well with the one calculated from known data of the tubular fluid concentration index ( $F/P$ ) for inulin along the proximal tubules suggesting indirectly that the flux per unit surface area remains constant during reductions of the luminal diameter.

According to Gertz an automatically adjusted glomerulo-tubular balance or load dependent proximal reabsorption rate would now be explained if any reduction in the glomerular filtration rate (or load) were to result in a reduced internal diameter and thereby in a reduced surface area. At a constant transcellular net flux per unit area the transfer rate would decrease *pari passu* with the filtered load. This hypothesis is clearly incompatible with some direct observations and physical facts. First of all it is in striking contrast to the author's own direct observation that the flux is constant along the accessible part of the proximal convoluted segment in which the flow rate (or load) is rapidly decreasing (about 60–75 per cent reabsorbed at the end of that part) indicating directly that the net flux of water and sodium is independent of the load (i.e. has the character of a  $T_m$  limited process above the saturation level). Furthermore it has been shown (Leyssac 1964 a) that the proximal internal diameters of the major part of the nephron population is uninfluenced by the rate of filtration within a great range of variation. Finally changes in the luminal diameter should by no means change the enormous surface area of the luminal brush border of the proximal tubules. The relevance of this physical fact was rejected by Gertz because it would imply according to the general concept that the proximal tubular lumina

would be emptied at reductions of the filtration rate within the physiological range of variation (in contrast to the observed facts) if the flux were constant per unit area. It was not taken into consideration that changes in the rate of filtration cannot occur physiologically without primary changes in the transcellular flux as might have been inferred from his direct evidence of a proximal salt  $Tm$ .

### b) *Kelman's hypothesis*

Kelman (1962) inferred from a theoretical treatment of micropuncture data a formal analogy between proximal reabsorption of sodium and water and the reaction velocity (or transfer rate) of a tubular catalytic flow reactor, in which the transfer rate is proportional to the fluid flow through the tubular lumen. The mathematical formalism describing the transfer kinetics in the proximal tubules in accordance with the model was based on the indication from the data provided by Walker et al (1941) viz that the fraction of the glomerular filtrate reabsorbed up to a given point in the proximal tubules is independent of the filtration rate as substantiated recently by similar direct experimental results (mentioned in Ch I B c). Further it was assumed that the proximal tubule is functionally uniform from the glomerular to the distal end (i.e. pars convoluta and pars recta) altogether implying that the volume of flow per unit of time past a given point in the proximal tubule decreases exponentially as a function of distance from the glomerulus. However, the entire deduction leading to the final conclusion that proximal reabsorption rate is linearly dependent on the filtration rate, and that reabsorption rate at any given distance from the glomerulus is reduced in proportion to the flow rate is based on the implicit assumption, the relevance of which is not even discussed, that the glomerular filtration rate is the independent variable. Certain results were deduced from the mathematical development of the model which does not include any suggestions of the possible mechanisms of a flow dependent reaction velocity in nephrons. These results apparently were not inconsistent with the experimental micropuncture data taking data of fluid to plasma ratios (F/P) of inulin from the early part of the distal tubule as representative of the fluid at the end of pars recta (Kelman 1962, fig 2). But it is highly questionable as also mentioned by Kelman whether the assumption is correct that pars recta is functionally equal to the convoluted part, since electron microscopic examinations (see Rhodin 1958) have revealed a decrease in microvilli and mitochondria density in the distal parts of the proximal tubules suggesting a decreased reabsorptive capacity in this segment of the tubules. Therefore it would be a more convincing support to the Kel

man hypothesis, if data obtained exclusively from the proximal convoluted segment were consistent with the model. Such data were recently reported by Lassiter Mylle and Gottschalk (1964) and Giebisch, Klose and Windhager (1964) who used saline diuretic rats. The F/P inulin obtained in proximal tubules at various distances from the glomerulus were interpreted as being consistent with the Kelman model. This model requires, of course that a plot of the inulin ratios versus distance from the glomerulus in a semilogarithmic system should yield a rectilinear curve starting at (1,0). In both reports the data might be fitted to a straight line, but this line clearly intercepts with the ordinate (log scale of F/P inulin) at a point significantly lower than unity. This fact however is not easily seen in the figure given by Giebisch et al (1964, fig 5), which may be misleading to the reader, since the ordinate value (1) is separated from the abscissa value (0) in such a way that the ordinate intercepts with the abscissa value (0) at a value far less than unity. Thus the directly obtained data are seen to be incompatible with Kelman's model as were also the ones obtained by Gertz (cf section a). Whether the data might fit the theoretical curve, calculated on the assumption of a constant transfer rate along this segment, was not discussed. However, a redrawing of the curve on the basis of their data makes it closely adaptable to such theoretical curve. Furthermore in the same paper Giebisch et al (1964 fig 1) showed a linear relationship between the percentile proximal fluid reabsorption and the distance from the glomerulus in isotonic and hypertonic NaCl loaded animals, in which the excretion fractions of sodium were less than 12 per cent of the filtered load. This demonstration which is in conformity with previously reported results from the same laboratory (Windhager and Giebisch 1961 b fig 3) and conclusions drawn by Walker et al (1941) from their data, indicates a uniform reabsorption rate along the convoluted part of the proximal segment.

These data obtained by direct methods are all inconsistent with the idea of a decreasing reabsorption rate along the proximal convoluted segment but greatly support the alternative - that the proximal reabsorption rate is independent of the load presented.

### c) *The general contemporary concept*

Generally the concept of gradient limitation of the proximal tubular reabsorption rates is still accepted in a slightly modified version. In his recent monograph, Pitts (1963) uses the term 'gradient time limitation' implying that rates of reabsorption of salt in the proximal tubules are determined by the gradients which can be established and by the time of contact of tubular



fluid with the tubular epithelium. The gradient is postulated to be established in the lower part (*pars recta*) of the proximal tubule (inaccessible to direct examination) and is of course purely speculative. At all events it seems to be a singularly unhelpful hypothesis, as it is quite safe to state that 60–70 per cent of the filtered volume has been reabsorbed at this lower part of the proximal segment. Thus any possible gradient for sodium here cannot possibly explain the fact that the sodium (or salt) and water reabsorption rates and glomerular filtration rate vary in almost direct proportion as apparent if seen from a purely hydrodynamic point of view (cf Ch I B d p p 23–24). The argument most often advanced against T<sub>m</sub> limitation of the proximal reabsorption rate of salt is the apparent fact that doubling the rate of filtration (e.g. in a dog fed with pure meat) would deliver to the tubules per min about twice the amount of salt they were able to reabsorb if the rate of reabsorption were T<sub>m</sub> limited and plasma sodium concentration were above the renal plasma threshold. The rate of salt excretion therefore should increase in proportion to the rate of filtration which is in contrast to the observed events. The argument is evidently fallacious because if the rate of proximal fluid reabsorption were T<sub>m</sub> limited the effective filtration pressure (and glomerular filtration rate) would never increase significantly under physiological conditions until the T<sub>m</sub> of salt and water (or volume T<sub>m</sub>) had changed.

## II INVESTIGATIONS BY INDIRECT AND DIRECT METHODS, BY WHICH TO ELUCIDATE THE PROBLEM

### a) *Indirect methods*

A variety of experimental procedures have been applied in clearance studies in attempts to investigate whether changes in the filtered load of salt is automatically followed by similar changes in the rate of reabsorption of the major fraction of the filtered load. Three main types of experiments may be distinguished and shall be discussed in brief.

It has been shown repeatedly in dogs (e.g. Selkurt and Post 1950) and in rabbits (Kruhoffer 1950) that elevation of plasma sodium concentration (and, thus in filtered load) by hypertonic sodium chloride infusion may give rise to an initial increase in glomerular filtration rate and rate of reabsorption of salt. By further elevation of the plasma sodium concentration a level may be reached at which sodium reabsorption rate remains constant or even decreases with increasing sodium concentration (above 165–170 meq/l) at a constant filtration rate. This type of experiment is of course extremely complex and

difficult to interpret, because relatively long periods of time (more than 10–15 min) elapsed between the induced change in load and the collection of urine for clearance determination. Secondary events, as nervous reflex mechanisms changing the renal blood flow and glomerular capillary hydrostatic pressure induced by volume change of the extracellular space changes in the aldosterone secretion changes in the oncotic pressure etc. may complicate a quantitative evaluation of the effect induced by the change in the sodium concentration as the only variable, as emphasized also by Kruhoffer (1960). Realizing this fact Bojesen (1954a and b) had previously stressed the importance of a study of the immediate effects of changes in the filtered load of salt and water rather than the steady states and drawn the full consequences thereof in his own experiments (cf Ch I p 24, and below). If anything the observation mentioned above might suggest that the rate of reabsorption of salt, at least at high sodium concentrations in the filtrate does not invariably change in parallel with the filtered load. A similar suggestion might be inferred from experiments in which hypertonic saline has been infused into one renal artery, the contralateral kidney serving as a simultaneous control thereby excluding some of the possible secondary effects. In this way e.g. Selkurt (1954) showed by step wise increasing the sodium concentration in the infused solution that a marked increase in sodium excretion rate electively could be obtained without measurable changes in the rate of filtration on the side of infusion. Simultaneous changes in the urine volume following these changes in the load however make the exact estimation of filtration rates highly difficult, since corrections for dead space errors were not carried out which to some extent obscured the significance of the observation. Apparently diametrically opposed results obtained in a series of clearance experiments in dogs, were reported by Hamm and Levinsky (1964). They calculated the rates of sodium reabsorption at different inulin clearances which were varied over a large range partly by pre loading with isotonic saline infusions and/or by desoxycorticosterone (and vasopressin) treatment partly by or combined with graded aortic constriction above the renal arteries. After a series of control collections the plasma sodium concentration was elevated by hypertonic saline infusion and the rates of reabsorption were determined at the various rates of filtration brought about either by partial closure or by a reopening of the aortic clamp. Now the rates of reabsorption at equal rates of filtration could be compared at high ( $>170$  meq/l) and low ( $<160$  meq/l) plasma sodium concentrations over a large range of variation in inulin clearances. They demonstrated that rates of sodium reabsorption at any glomerular filtration rate thus effectuated were significantly higher at the high sodium concentration level than at the low level. Further

more the amount of sodium reabsorbed per 100 ml of filtrate increased with increasing plasma sodium concentration in the range 140 to about 190 meq/l. Since no such difference between animals with and without desoxy corticosterone treatment was observed, secondary changes in the adrenal cortical activity could be ruled out as a reason for this apparent paradoxical observation. The somewhat more complicated procedure (aortic constriction) a demonstration of a decrease in the plasma protein concentration on loading and the fact that collections were not made until at least 45 min after start of the hypertonic loadings involves that the interpretation of the increase in the reabsorption rate of sodium at high plasma sodium levels become rather difficult in these experiments. It was suggested that the sodium reabsorption might have changed merely as a direct effect of the increased sodium load. This suggestion was by no means established by their data. Assuming that the proximal tubular reabsorption of salt has the character of a Tm process under intrarenal hormonal control the observation of the relatively increased sodium reabsorption rate after hypertonic loading the glomerular filtration rate being reduced by aortic constriction to any particular value identical to a value in a pre-loading period, might suggest merely that a normal balance between glomerular propulsive pressure and the controlled Tm is disturbed under these conditions. Any explanation of the seemingly opposed results obtained after hypertonic saline loadings cannot be given until the mechanism(s) by which the hormone release is effectuated have been outlined in greater detail; the results might even eventually contribute to a future understanding of such mechanisms.

The interpretation of experiments on mechanically changed diuresis may be less complex. Shipley and Study (1951) studied the effect of an acute elevation of perfusion pressure in the renal artery of a semi isolated kidney in anaesthetized dog. By means of a pumping device blood drawn from a carotid artery of the same dog could be pumped into the renal artery at any desired pressure. They showed that increasing pressure within the range of 80 to 180 mm Hg (the autoregulated interval of pressure) was followed by marked diuresis and saliuresis without any appreciable increase in the glomerular filtration rate or the renal plasma flow. Conversely in acute experiments minor reductions in the renal arterial pressure, induced by partial clamping of the renal artery may reduce significantly the rate of salt excretion on that side without any measurable change in the filtration rate as demonstrated by Blake et al (1950) and Sellkurt (1951 a). This fact could also be demonstrated over prolonged periods of time thus excluding dead space errors as an explanation (Mueller et al 1951). A reasonable interpretation of the results obtained from this type of experiments would be that induced changes

in the renal arterial pressure have changed the glomerular propulsive pressure to some extent in the same direction without concomitant, parallel changes in the proximal rate of reabsorption. Hence similar changes in the proximal intratubular pressure have occurred which explain satisfactorily the observed changes in the rate of salt excretion as well as the unchanged rates of filtration (unchanged effective filtration pressures). This was actually how Selkurt interpreted these results (Selkurt 1951 b), viz that the experimental results suggested a  $Tm_{Na}$ . As parallel changes in the glomerular filtration rate and the sodium reabsorption rate were seen to occur, however at major reductions in the renal arterial pressure and since it could not be excluded in the former experiments that changes in the salt excretion might have been due to changes in the distal reabsorption rate the idea of a proximal salt  $Tm$  could not be considered established and was given up.

The third type of experiments are those in which the glomerular propulsive pressure is acutely changed by rapid infusions of large amounts of saline solutions containing either high concentrations of proteins or no colloids at all (dilution diuresis). Rapid and significant changes in the oncotic pressure may be induced in this way but because of instantaneous and considerable changes in the urine flow the sequence of events and rapid changes in glomerular filtration rate cannot possibly be detected and quantitatively evaluated unless careful corrections for dead space errors are undertaken. To the author's knowledge such corrections have not been applied to this type of experiments except by Bojesen (1954 b) (referred to in Ch I B d p 24) for which reason his experiments exclusively shall be commented here. This author showed that an acute lowering of the oncotic pressure even by 8 mm Hg was followed by an instantaneous rise in the urine output and salt excretion rate the glomerular filtration rate the rate of reabsorption and the renal plasma flow remained unaffected until about 10–15 min after the induced change in the glomerular propulsive pressure. Within this time interval a direct proportionality was observed between the changes in plasma oncotic pressure over a range of about 8 mm Hg and the changes in the rate of water and salt excretion (Bojesen 1954 b fig 2). Furthermore it was demonstrated that the composition of the urine excreted in excess after the oncotic pressure had been lowered corresponds fairly well to that of an ultrafiltrate as regards chloride and total cations. Therefore it was concluded in accordance with the interpretation of results from mechanically evoked diureses mentioned above that salt is reabsorbed as a  $Tm$  substance probably throughout the entire nephron. Consequently this indirect evidence would infer as emphasized by Bojesen and also in the present review (Chapter I) that the rate of filtration is determined by the rate of reabsorption in the proximal

tubules i.e. by the actual value of the  $T_m$  at that very moment. Since the inulin clearance and renal plasma flow had increased 10–15 min after the infusions (II phase) whether or not the solutions contained proteins, Bojesen suggested that some humoral factor(s) presented to the cells might have changed the reabsorptive capacity. The nature of such substance(s) remained undetermined nor could the reason be established of the prolonged but moderately increased salt excretion rate observed 45–60 min after such volume expansion ("III phase"). The only relevant argument against Bojesen's conclusions seems to be that it could not be excluded here that the process of reabsorption might have been inhibited in some way (e.g. by the change evoked in the oncotic pressure or in the intratubular hydrostatic pressure proper) to exactly the same extent as the reabsorption might have been increased by the elevation in the filtered load. Obviously this possibility is highly speculative and would seem less likely.

Altogether the possibility of an existence of a rate limiting proximal salt  $T_m$  is not precluded in any of the results obtained in these clearance studies. If anything they might be suggestive of such concept, or as seen from Bojesen's results only, provide rather strong evidence hereof.

In order to understand the physiology and pathology of the kidney it is of obvious and major importance to know whether the proximal tubular salt transfer is  $T_m$  limited or load dependent. Hence it is rather amazing that later investigators apparently have neglected to take into consideration this problem even the evidence presented by Bojesen has been dismissed and it has been declared that the validity of his dead space error corrections remains to be confirmed (e.g. Wesson 1957, p. 320), although the same author acknowledges the value of Bojesen's method by which it has become possible to measure clearances of inulin when urine flows is variable (Wesson 1957 foot note p. 282). If, actually, later investigators had accepted the question as a problem of importance all efforts would primarily have been focussed on the confirmation or disapproval of the validity of Bojesen's method and results or using the direct methods might have attacked the problem as such. For several years such efforts were not mobilized in the field of renal physiology and gradually this problem has faded off. Most authors interpret without further ado their results according to the theories advanced by Smith while Krühoffer (1960) apparently has accepted the existence of a rate limiting proximal  $T_m$  for salt and water. In his review Krühoffer pays the major attention to hydrodynamic considerations in an interpretation of the results (viz. possible changes in the glomerular propulsive pressure and resistance to fluid flow along the tubular lumina). This point of view seems to be of more relevance when the extent to which changes in the proximal reabsorp

tion rate as an isolated event, may change the intratubular pressure and the glomerular filtration rate without disturbing the sodium excretion and when the consequences of isolated changes in the glomerular propulsive pressure have to be evaluated. Since these questions are of secondary importance only to the crucial point whether or not the proximal reabsorption rate is load dependent being relevant only in the case of load independency major attention has been paid to this latter problem in the present study. Further hydrodynamic considerations on the pressure decline across the glomerular membrane are apparently of minor importance when  $\pi$  proximal salt  $T_m$  or volume  $T_m$  is a variable parameter and physiological variations of the  $T_m$  are part of a mechanism controlling the proximal intratubular pressure.

#### b) *Direct methods*

An approach to the problem by direct methods permitting an estimate of the tubular function independent of the simultaneous rate of filtration might be possible by the micropuncture technique in its advanced technical perfection. It is technically possible by the micropfusion device, described by Sonnenberg and Deetjen (1964) to perfuse at any desired, but constant perfusion rate within the range of  $10-35 \times 10^{-6}$  ml/min (normal flow rates in proximal tubules of the intact mammalian kidney) into a proximal tubular segment, separated from the glomerulus by an intraluminally injected column of oil and to collect samples for ultra microanalysis of the perfusion fluid at a more distal site. By this technique the rate of reabsorption may be determined at different tubular loads independent of the overall functional state of the kidney and possibly without significant interference with an intrarenal regulation mechanism(s) if any. This recently developed method has not yet been applied to investigations of this type. Some fundamental informations, however, are obtainable from the conventional micropuncture technique by analyses of samples collected at various later determined distances from the glomerulus, as already mentioned (section A a and b in the present chapter). The value of this conventional technique is limited particularly as regards the problem here discussed, whether the observed *rapid changes* in the state of tubular function may be due primarily to changes in glomerular vascular factors or to simultaneously occurring changes in the renal blood flow cannot be decided on the basis of results thus obtained.

It was of great interest when Hanssen (1960) provided evidence that fluid in the proximal tubular lumina at the time of interruption of the renal circulation (and filtration)  $\pi$  continuously reabsorbed into the interstitial space

until the convoluted part (4/5 of the proximal tubule) is emptied. Ferrocyanide, known to be excreted by filtration only, was injected intravenously into mice *in vivo*. The left exteriorized kidney was subsequently frozen at the moment of sacrifice. 5 sec after the injection, the intraluminal distribution of ferrocyanide, precipitated as Prussian blue, was examined in nephrons isolated by microdissection. The distribution of ferrocyanide in tubules of the left snap frozen kidney was compared with the distribution in the tubules of the right kidney, frozen at different, stated time intervals (20-300 sec) after the animal was sacrificed. It was observed that ferrocyanide in the upper 4/5 of the proximal tubules was not displaced to any significant degree and did not pass the bend of the loop of Henle. A displacement in a proximal direction was never observed. Furthermore, the intertubular space in kidneys, the renal pedicles of which had been tied before freezing, were seen to be very wide at the time when the proximal tubules were occluded, in contrast to a narrow intertubular space seen in kidneys frozen at the moment of interruption of the blood circulation, which observation has been confirmed by the present author's experiments (cf. Leyssac 1965 a, figs 4 and 5). Consequently, a fair agreement could be anticipated between the tubular fluid content and the amount of draining fluid collected from the renal vein after removal of the kidney, because some intertubular space still remains in the cortex of a drained kidney. Such fair agreement was actually observed. Swann (1960) using rats found a value of 0.230 ml/g of moist tissue drained from the kidney; this value was confirmed by Little and Robinson (1963) who found that 0.217 ml/g of moist tissue actually was drained, and 0.248 ml/g of moist tissue was lost as deduced from analyses of tissue frozen *in situ* and after exsanguination. These latter investigators demonstrated also that the composition of the extra fluid lost from the kidney upon exsanguination resembled extracellular fluid (139 meq  $\text{Na}^+$ , 127  $\text{Cl}^-$  per kg of water, but no considerable amounts of  $\text{K}^+$ ). These values agree with the volume of proximal tubular fluid in rats viz. 0.18-0.19 ml per kidney in animals with a kidney weight of an average of 0.87 g (equal to 0.21-0.22 ml/g of tissue) calculated by Leyssac (1963) on the basis of the known number of nephrons and dimensions of the lumen. It would be justified to state on the basis of these arguments that the proximal luminal occlusion observed in the early period following interruption of the renal circulation is due to a continued reabsorption. Consequently, the rate of disappearance of the lumina measurable *in situ* in the rat by *in vivo* microscopy, might serve as a measure of the rate of proximal reabsorption (i.e. of the volume of proximal fluid reabsorbed per unit of time and unit of tubular length). Provided that the composition of the tubular fluid and the volume of fluid per unit of tubular length of the

proximal segment ( $r =$  the internal diameter) is kept reasonably constant from one experiment to the other, a difference in the recorded time interval (denoted as 'occlusion time') from interruption of the circulation until the major part of the proximal lumina has disappeared would indicate directly a difference in the rate of transfer of salt and water. By this method described by Leyssac (1963), it was observed that the lumina were occluded on an average 18.5 sec after interruption of the renal circulation at inulin clearances of the lower range (about 1 ml/min/g  $\pm$ W) which was the state of function most frequently observed in the laparotomized and moderately saline loaded animals used here (body weight av. 250 g) (whole material Leyssac 1965 b). The *in vivo* observation was confirmed by the histological findings of almost completely occluded proximal tubules in kidneys frozen 18 sec after removal from rats under similar experimental condition (Leyssac 1965 a fig. 5 and Steinhausen et al. 1963) the latter authors microphotographed at rapid successive exposures the kidney surface in antidiuretic rats after interruption of the renal circulation. In order to make the lumina contrast the bright cellular lining and brush border Lissamin green was administered intravenously prior to the arterial clamping. By this technique Steinhausen showed that the proximal lumina in rats were occluded within 20 sec i.e. the occlusion time most frequently seen in antidiuretic and moderately diuretic rats. The fairly good agreement between reabsorption rates of salt and water in rats evaluated by measurements of the occlusion time and by other methods would establish that the occlusion time method may be used for estimations of the proximal reabsorption rate. It was found that the occlusion time to be expected if calculated from the immediately preceding clearance of inulin (and on the assumption that the proximal reabsorption continues at an unchanged rate until the lumina were emptied) was in rather close agreement with the rate actually measured over the entire range of spontaneous variation (e.g. av. 16.5 sec calculated and av. 18.5 sec observed at a clearance of 1.0 ml/min/g  $\pm$ W, Leyssac 1963, 1965 b). Agreement between these two independent methods by which to estimate the rate of proximal reabsorption in the same animal and at the same time was found to be equally satisfactory in young animals (body weight 100 g) in which dimensions of the nephrons are much smaller than in adult rats (Leyssac 1965 c). A similarly fair correspondence is found even if related to reabsorption rates in the proximal tubules of rat kidneys, as reported by other investigators using other direct methods. With an internal radius of 12.5  $\mu$  (a value between the ones obtained histologically in snap frozen kidneys by the author and the ones obtained *in vivo* by Steinhausen et al. (1963) (cf. Leyssac 1965 c)) together with a plasma sodium concentration of 150 meq/l  $\pm$  1  $\mu$  thick cross section of a proximal



tubule will contain  $7.35 \times 10^{-8}$   $\mu\text{eq Na}$  in  $490 \mu^3$  of luminal fluid. With an occlusion time of 18.5 sec and an external radius of  $17.5 \mu$  (deduced from the observation of a transected cellular area to luminal area ratio about unity (Leyssac 1965 c)),  $3.6 \times 10^{-5}$   $\mu\text{eq/mm}^2 \text{ sec}$  are found to be transported across the peritubular membrane or  $5 \times 10^{-5}$   $\mu\text{eq/mm}^2 \text{ sec}$  transported across the luminal membrane (considering the lumen as a cylinder with a radius of  $12.5 \mu$ ). This value may be related to the value of  $3.0 -$  and  $3.4 \times 10^{-5}$   $\mu\text{eq/mm}^2 \text{ sec}$  reported by Windhager and Giesbisch (1961 a) estimated on the basis of data obtained by the stop flow microperfusion technique in single proximal tubules of rats and by short circuit current measurements, respectively, and to the value of  $8 \times 10^{-5}$   $\mu\text{eq/mm}^2 \text{ sec}$  obtained by Gertz (1963) using another modification of the micropuncture technique, referred to previously (p. 52-53).

The close agreement between the observed occlusion time and the one calculated from the immediately preceding clearance of inulin on the assumption of a constant and unchanged reabsorption rate after interruption of the renal circulation and filtration indicates that the reabsorption actually does continue in the proximal convoluted segment at a constant rate until no filtrate is left within the lumen, a direct observation permitting the conclusion to be drawn that the rate of reabsorption is independent of the filtered load per se (Leyssac 1963).

Based on the same assumption the relationship between reciprocal occlusion time and inulin clearance per g of kidney weight ( $\text{K.W.}$ ) should remain unchanged with uniform fractional reabsorption proximally and uniform total fluid volume of the proximal convoluted segment per g  $\text{K.W.}$  ( $\frac{V}{\text{K.W.}}$ ). Data obtained in experiments using small rats (100 g body weight) (Leyssac 1965 c) indicated that this relationship did not differ significantly from the ones seen in adult rats. Accepting that the fractional proximal reabsorption of filtrate is about the same in young and adult animals,  $\frac{V}{\text{K.W.}}$  should be equal in these two groups of rats always provided that the basic assumption is valid. It was observed that the ratio cross sectioned proximal luminal area to cellular area was approximately the same in the two groups and that the luminal volume per unit of length was proportional to the kidney weight  $\frac{V}{\text{K.W.}}$  therefore would be equal in the two groups if the length of the proximal tubules is equal in 100 g rats and 250 g rats. This is a reasonable assumption as it has been shown by Fetterman et al (1964) by microdissection that the final step in the development of the proximal convoluted tubules in man in the age group 15-21

years is an increase in the volume without further increment in the segmental length. In children at the age of 15 years the proximal volume was found to be at an average of  $0.0423 \text{ mm}^3$  as opposed to an average volume of  $0.0875 \text{ mm}^3$  in adults. In accordance with this the kidney weight was twice as high in adult rats as in rats of 100 g. Thus the data obtained in small rats supports the conclusion that the rate of reabsorption continues at an unchanged rate during the luminal occlusion period after cessation of the glomerular filtration independent of the load.

Thirdly, the fact that the great majority of the proximal convoluted tubules in a field of vision complete the luminal occlusion within plus or minus 2 sec may possibly be taken as additional evidence in the discussion of a load dependency of the proximal fluid reabsorption and may further serve as basis for a brief comment on the limitations of the occlusion time method. The fairly well defined end point of luminal occlusion of the visible two thirds of the proximal tubules might apparently suggest that fluid reabsorption is completed almost simultaneously along this segment since the luminal fluid is not displaced to any significant degree either distally or proximally according to arguments discussed above (p. 62). Since the flow rate of luminal fluid (and thus the load) is rapidly decreasing almost exponentially along this tubular segment because of reabsorption, an equal rate of fluid reabsorption here would indicate that the proximal reabsorption rate is independent of the load. A quantitative estimation however will disclose that the measured occlusion time *alone* does not permit this conclusion. Thus it might be postulated that the distribution of proximal internal diameters in control rats (cf. Leyssac 1964a, fig. 7) represents the distribution of diameters along equally sized but conical segments in such a way that the major group of measured diameters (about  $27 \mu$ ) represent the diameter of the middle half of the convolution, the group with larger diameters (about  $32 \mu$ ) represents the diameter of the early one fourth of the segment, and the group with small diameters (about  $22 \mu$ ) the distal fourth of the convoluted part. Moreover, if it is postulated that an occlusion time about 18 sec (16–20 sec) represents a mean of different occlusion times along the segment with the early part occluding first (in 16 sec) and the distal one fourth last (in 20 sec), it will appear that the rate of fluid reabsorption decreases almost exponentially (from about  $3.2 \times 10^{-4} \text{ mm}^3 \text{ mm}^{-1} \text{ sec}^{-1}$  to  $1.75 \times 10^{-4} \text{ mm}^3 \text{ mm}^{-1} \text{ sec}^{-1}$ ) and parallel to the rate of fluid flow along this segment. It therefore must be concluded that measurements of the occlusion time are too inaccurate *per se* to exclude the possibility of an exponential decrease in the reabsorption rate along the proximal convoluted segment. Stemhausen et al. (1963) observed however in photographic serie-exposures that the luminal occlusion

proceeded from the distal end of the proximal convolution towards the glomerulus. If this fact is included in the above considerations (always maintaining the postulate of the conical shape of the segment) it would mean that the distal parts occlude in 16 sec and the early parts in 20 sec. Then the rate of reabsorption will appear to remain almost unchanged along the convoluted segment ( $2.5 \times 10^{-4} \text{ mm}^3 \text{ mm}^{-1} \text{ sec}^{-1}$  in the early part as opposed to  $2.2 \times 10^{-4} \text{ mm}^3 \text{ mm}^{-1} \text{ sec}^{-1}$  in the distal part). Finally it seems more reasonable if the distribution of the internal diameters is considered to be representative mainly of diameters in nephrons of unequal sizes. It may then be concluded that the fairly well defined end point of the luminal occlusion in the major part of the visible convolutions actually excludes the possibility of decreasing reabsorption rate along the proximal convoluted segment, the additional information provided by Steinhilber's observation being taken into account. In other words the well defined end point of occlusion serves as further direct evidence of the feature that the proximal reabsorption rate is independent of the load. Finally it should be borne in mind that data obtained by two different micropuncture techniques — Gertz flux measurements and determinations of F/P inulin ratios along the proximal tubule —, although differently interpreted by the authors (cf section A a and b of this Ch) clearly had indicated that the proximal reabsorption rate along this segment is independent of the load, in accordance with the latter conclusion drawn from the occlusion time method.

It should be emphasized however that even if direct measurements had shown that the proximal reabsorption rate decreases exponentially along the segment as opposed to the demonstrated fact, such evidence need not at all have invalidated the concept of  $T_m$  limitation of the rate of fluid transfer across the tubular cell. The demonstrated close agreement between the occlusion time calculated on the preceding inulin clearance and the one directly observed (cf p 63) representing the occlusion time of a tubule section with the mean diameter indicate beyond doubt that the local fluid reabsorption (across the single cell) continues at an unchanged rate during the occlusion period independent of the load. Naturally this fact has not excluded a priori that different (decreasing) transport capacities (or  $T_m$  levels) might have existed along the proximal convolution. However observations by three independent methods (1) by the flux measurements, 2) the F/P inulin ratios, and 3) the well defined end point of occlusion) that the rate of fluid reabsorption along the proximal convoluted segment is independent of the load presented may be characterized as additional evidence of the concept of  $T_m$  limitation of this rate.

The existence of spontaneous variations in this rate indicates that the

$T_m$  is variable, at least within this range. Moreover, below the spontaneous minimum value the rate of reabsorption is not changed significantly (Leyssac 1964a fig 2), as estimated from the occlusion time, by reductions in the rate of filtration even not if the inulin clearance is reduced far below the spontaneous range of variation of this parameter, e.g. by partial clamping of the renal artery. The requirements of uniform composition of the luminal filtrate and identical internal diameters in the major part of the proximal tubules were apparently fulfilled in these experiments. It may be questioned whether the skew distribution of internal proximal diameters in these clamped kidneys including many tubules of small calibre (cf Leyssac 1964a fig 7) may have affected the registered end point of occlusion thus rendering it less well defined. This possibility cannot be excluded and probably it may become of significance at the very low clearance values (below 0.5–0 ml/min/g K.W.) thus involving that the occlusion time will be too brief. But the method being used as and only permits, a rather rough estimate of the proximal reabsorption rates this objection cannot invalidate the conclusion that the proximal  $T_m$  for salt and water has reached a minimal fixed value at rates of filtration below the minimal spontaneous value, depressed (artificially or pathologically) by a lowering of the glomerular propulsive pressure. At these low clearance values the proximal salt- or volume-  $T_m$  is invariable like the glucose  $T_m$ . At these low rates of filtration right up to the minimum normal rate reductions in the filtration rate (or more correctly in propulsive pressure) should bring about tubular occlusion (cf the argument by Gertz Ch III A p 53–54). Actually it was found that below this rate of filtration the urine flow would decrease in direct proportion to the inulin clearance indicating that a decreasing number of nephrons participate in the urine formation *passu* with decreasing filtration rates. Besides occluded proximal tubules were histologically demonstrated in kidneys frozen at this state of function as opposed to the wide open lumina seen in all tubules at higher clearances. The demonstration of a proximal luminal occlusion at these low rates of filtration represents a further (fourth) demonstration of the proximal  $T_m$  limitation of the fluid reabsorption rate.

The conclusion to be drawn is that investigations by direct methods have definitely shown that under normal conditions the rate of proximal tubular fluid reabsorption is  $T_m$  limited in mammalian kidneys.

As the proximal fluid reabsorption has the character of a bulk reabsorption (cf Ch II p 41) and since variations in the reabsorption rate apparently are variations in the transfer rate of a volume of an aqueous solution of electrolytes and urea it follows that reabsorption rates not only of sodium but also of chloride, bicarbonate, potassium and urea apparently are limited

by one and the same step in the transfer process. It may surprise that this fact (Tm limitation) has escaped recognition and discussion in most of the previous investigations. One explanation may be that the Tm apparently is rapidly variable which has not been considered (at any rate not been expressed) except by Bojesen apparently because the concept of Tm has arisen from and later in general been attached to, a specific method used in a particular experimental situation, by which method such rapidly variable Tm is not demonstrable. Bojesen seems to be the only previous investigator who has designed experiments by other methods in order to demonstrate (or disprove) a Tm process of this type. This deranged way of thinking has also impeded the interpretation of such micropuncture data which might have provided some evidence. It even prevented the authors from analysing the untraditional possibility of a variable Tm on the basis of their own data. It seems rather irrelevant to define theoretically any concept on such criteria. It is generally agreed that the distal transfer capacity of sodium is Tm limited and that the capacity may be changed by the action of mineralocorticoid hormones within 45 to 60 min. Although the conventional clearance method fails to demonstrate changes in the proximal Tm occurring in less than 20–30 min it is by no means justified to infer that the proximal transfer process *on principle* cannot be Tm limited. The criterion is not whether the rate of transfer may be changed in 40, 20, 10 or one min. The time interval necessary for a change to occur is solely determinant for the methods by which the Tm character may be demonstrated.

As a consequence of the above presented arguments the concept would arise that there is *on principle* no difference between the process of reabsorption of salt (or sodium) in the proximal and distal segment of the mammalian nephron. In both segments the process is Tm limited and may be varied rapidly in the proximal segment and more slowly in the distal segment.

If this solution of the problem concerned (the problem of load dependency versus Tm limitation of the proximal reabsorption rate of salt and water) is now accepted as the only reasonable conclusion to be drawn from the data presented it may be seen already that it has a great bearing on the interpretation of changes occurring in the rate of glomerular filtration.

## CHAPTER IV

### Consequences of $T_m$ -limitation of proximal reabsorption of salt and water

#### A THE CONCEPT OF THE GLOMERULAR FILTRATION RATE

It was previously argued (Ch I § d p 23-25) that an increase in the glomerular propulsive pressure would be accompanied by a similar increase in the proximal intratubular pressure unless either the tubular resistance to flow was reduced or the rate of proximal reabsorption was increased. This statement was based on observations in rats under normal non diuretic conditions under this condition a pressure difference of 12-13 mm Hg was found between the end of the proximal convoluted segment and the renal pelvis through which distance no more than 20-25 per cent of the filtered fluid volume is propelled. If the resistance across the glomerular membrane were equal to the resistance offered by these distal segments of the nephrons a pressure drop (i.e. an effective filtration pressure) of at least 50-60 mm Hg would be required to account for the actually filtered volume. With a mean oncotic pressure in the glomerular capillaries of about 30 mm Hg the hydrostatic capillary pressure should be at least 80-90 mm Hg which of course is unrealistic. No doubt the resistance to flow across the glomerular membrane must be less than the resistance offered by the nephron. The actual effective filtration pressures however, remain unknown. Pappenheimer (1955) tried to calculate the filtration coefficient (ml filtered per min per mm Hg effective filtration pressure) from observations on the glomerular sieving of myoglobin and ovalbumin in dogs. A hypothetical pore radius and the filtration coefficient can be calculated by substituting the known diffusion coefficients, the Einstein Stokes radii of the protein molecules and the restriction of filtration of these proteins ( $C_0/C_1$ ) in the sieving equation

$$\frac{C}{C_1} = \frac{1 + \frac{D}{Q_f} \times \frac{8\eta K_f}{r}}{1 + 2.4 \frac{a}{r}} + \frac{D}{Q_f} \times \frac{8\eta K_f}{r^2}$$

$$2(1 - a/r)^2 - (1 - a/r)^4$$

in which (a) denotes the radius of the protein molecule (r) the pore radius ( $Q_f$ ) the filtration rate (ml/min) (D) the diffusion coefficient ( $\eta$ ) the

viscosity, and ( $k_f$ ) the filtration coefficient. By substituting the Einstein Stokes radii a spherical shape of the molecules has to be assumed. However, neither myoglobin nor ovalbumin molecules are spherical, nor were corrections for electrical hindrance due to negative charges of the proteins included in Pappenheimer's calculation. Thus the pore diameter of 75 Å calculated for dog glomerular membranes is probably an underestimation. Moreover, as the pore radius in the fourth power (Poiseuille's law) determines the filtration coefficient, the estimation of the latter is probably also somewhat underestimated and was found to be on an average 3.1 (1.9–4.5) ml per min per mm Hg per 100 g of kidney weight (KW). Based on a filtration rate in dogs of about 1 ml per min per 100 g KW a pressure drop across the glomerular membrane of 20 mm Hg ( $\Delta P$ ) was calculated from

$$\Delta P = \frac{Q_f}{k_f}$$

From similar clearance experiments in dogs using a dextran preparation including molecules of different molecular weights Wallenius (1954) determined in each sample the distribution of the different molecular weights (compiled in groups with an interval of 4 000) and calculated the renal clearance of each molecular class expressed as a percentage of the simultaneously determined clearance of creatinine (Wallenius 1954 Table 12). By relating the clearance of each group to the representative molecular weight (Fig. 20) a curve was constructed which approached zero filtration at a molecular weight of about 50 000, corresponding to an Einstein Stokes radius of about 50 Å. The advantage of using dextran is that these molecules are uncharged and no electrical hindrance occurs. Hence a pore diameter of about 100 Å might probably be a more correct value for the calculation of the filtration coefficient. Using these results Wallenius and Garby (quoted from Kruhoffer 1960 p. 264) calculated a pressure drop of only a few mm Hg across the glomerular membrane. Wallenius' data, however, are subject to the objection that the dextran fractions were not quite homogenous although they precipitated as representative of an absolutely distinct fraction. Thus the determination of the low concentrations of high molecular weights are questionable and probably underestimated.

Of course estimations like those mentioned above should be considered with a certain reservation because of the many unestablished assumptions underlying the calculation. One of the serious objections has been that pores in the basement membrane of capillaries could not be demonstrated by electronmicrographs using the conventional techniques although this structure was found to constitute the main filtration barrier to ferritin molecules (mole

cular diameter about 100 Å) in rat glomeruli (Fahrquhart, Wissig and Palade 1961). Therefore it may be of interest that Faarup and Christensen (1965) were able to demonstrate a porous structure of the dense layer of the basement membrane of peritubular capillaries in the medulla of rat kidneys in freeze dried specimens frozen at the moment of removal of the kidney, this procedure might give a more true picture than the one obtained by the conventional technique at which tissues are fixed several minutes after cessation of the organ function. The parallel pores perpendicular to the membrane surface, had a diameter of 100–300 Å. Still it remains to be established however, whether or not this observation might be the result of an artifact due to the applied technique.

Were any significance to be attached to these indirect estimations the resistance to fluid flow across the glomerular membrane would apparently be inferior to the resistance offered by the distal tubular segment (from the end of the proximal convolution and throughout). Since the  $T_m$  limitation of the proximal reabsorption rate of salt and water is now established the proximal intratubular hydrostatic pressure should vary as a primary event almost parallel with changes in the glomerular propulsive pressure. The indirect observation by Bojesen (1954b) and the direct observation by Gottschalk and Mylle (1956), mentioned previously (Ch. I, B. d), that such parallel changes do occur immediately after induced changes in the propulsive pressure may be considered a substantial experimental support to the main conclusion drawn from the above calculations. *It follows that significant changes in the effective filtration pressure (or filtration rate) without violent changes in the excretion rate of salt and water (due to a reduced resistance to flow) will not occur until some factor(s) has changed the  $T_m$  of the proximal reabsorption. Detectable changes in the rate of filtration are therefore reflections of primary changes in the proximal reabsorption rate of salt* and measurements of the inulin clearance should not be considered as any measure of the hydrostatic pressure level in the glomerular capillaries as determined by afferent and/or efferent arteriolar tone (i.e. of glomerular propulsive pressures) rather such measurements indicate the intrarenal turnover rate\*) of salt and water so long as the excreted fraction remains small. The realization that turnover rates of salt and water in the range of physiological variation are determined by the rates of proximal reabsorption and that these rates represent different states of a variable  $T_m$  process has an important implication to the interpretation of clearance data. In this realization is evidently implied that even though the clearance of inulin

---

\*) In this context the term 'turnover' is defined without reference to the pool concept.



might be determined exactly without any error and variations at all within the collection period it remains theoretically impossible to deduce from measured clearances and excretion rates of water and predominant electrolytes whether changes in the rates of excretion are caused by changes in the glomerular propulsive pressure or by changes in the tubular reabsorption rates. The correctness of this statement is apparent from the fact that the rates of excretion are determined not only by the effective filtration pressure and the overall rates of reabsorption but also by the tubular flow resistance and the distribution between proximal and distal reabsorption. Because of its importance to the interpretation of clinical and pathophysiological studies in human subjects this statement shall be illustrated in some detail.

By way of example a condition of reduced inulin clearance and greatly increased excretion rate of salt may be visualized. The overall rate of reabsorption would be clearly reduced. 1) This condition might be brought about at an unchanged glomerular propulsive pressure and tubular resistance in which case the proximal as well as the distal reabsorption rate are decreased. Because of the decreased proximal reabsorption rate the proximal intratubular pressure would be increased (lowering the effective filtration pressure) and the flow rate to the distal segments would increase. If the distal reabsorption rate also is assumed to be relatively more reduced than that in the proximal segment the rate of excretion would be increased not only because of the overall reduction in the transfer rate but also on account of the changed distribution between the proximal and distal reabsorption. 2) A similar clearance and excretion pattern might be observed in a condition in which the glomerular propulsive pressure is moderately augmented and the proximal and distal reabsorption rates of fluid are decreased relative to the same degree. The fraction of the filtered volume excreted would then be somewhat greater than in the former condition and the increased excretion rate of salt would be attributable partly to the change in the propulsive pressure partly to the change in the tubular function. 3) Finally an increased excretion rate of salt in a state of reduced filtration rate might also be obtained in cases in which the propulsive pressure is reduced and the overall reabsorption rate decreased in such a way that the proximal reabsorption rate is greatly inhibited and the distal reabsorption rate is increased. In this case it might be assumed that the resistance offered by the loop of Henle is reduced or abolished. Then the proximal and distal intratubular pressures might approach each other at a value a little above that normally found in the distal tubules (e.g. 10 mm Hg) and the effective filtration pressure might still be reduced because of the reduction in the glomerular propulsive pressure. The part played by the distal reabsorption in the overall reabsorption would be relatively increased and the

increased rate of excretion of salt would be the resultant of a decreased propulsive pressure decreased resistance to flow and a changed distribution between proximal and distal reabsorption

It may also be worth while to consider a condition of a greatly impaired and unchangeable tubular reabsorption throughout the whole nephron. At a normal glomerular propulsive pressure (and unchanged resistance) the rate of filtration will be reduced. If now the glomerular capillary hydrostatic pressure is increased (e.g. by afferent arteriolar relaxation) the rate of excretion will also be increased and the augmentation will correspond to the actual increase in the rate of filtration. In such a condition the rate of filtration might reach a normal value at a state of violently increased excretion rates of salt and water which might possibly amount to even more than 50-60 per cent of the filtered volume. The intratubular pressures and resistances in the nephron would of course, be highly disturbed in this imaginary case which cannot be compared with normal conditions.

Finally it should be mentioned also that since determinations of the turnover rate of salt by the clearance method in its usual performance invariably involves a variability of 5-6 per cent or more between the individual collection periods it may theoretically be observed in human subjects in whom the inulin clearance is unchanged that the rate of excretion of sodium may change within plus or minus 700-800  $\mu\text{eq}/\text{min}$  (at a plasma sodium concentration of 140  $\text{meq}/\text{l}$ ) solely as a consequence of changes in the glomerular propulsive pressure i.e. at an absolutely unchanged rate of sodium reabsorption.

In view of the present concept that significant changes in the effective filtration pressure in the individual nephrons (i.e. changes corresponding to measurable changes in the rate of filtration of the entire kidney, if parallel changes occurred in all the nephrons) normally are mediated only by changes in the proximal reabsorption rate of fluid some rigidity of the proximal tubular wall has to be assumed. Otherwise moderate changes in the filtered load would be followed only by a corresponding change in the tubular diameter with no appreciable change in the intratubular pressure and moderate changes in the intratubular pressure and thereby in the effective filtration pressure induced by primary changes in the rate of reabsorption could not be established. It might be questioned whether or not the proximal tubules actually do behave normally as more or less rigid tubules. Gottschalk and Mylle (1956, 1957) showed that the proximal intratubular pressure always was almost identical with the simultaneously measured peritubular capillary pressure in the same kidney in control (i.e. non diuretic) rats and these pressures increased in parallel within a range of 10 to 50 mm Hg no matter whether this

pressure increase was induced by renal venous compression, ureteral occlusion, or osmotic diuresis. This observation might suggest that the tubular wall would give way to an increase in the pressure from the outside as well as from the inside of the tubule until identical pressures were obtained in the interstitial space (capillaries) and the lumina. The applied pressure changes, however, exceed by far the physiological interval of pressures and although the results indicate that the suggestion largely is valid for induced, considerable changes in the pressures they fail to give any information whether or not this might also be true within the physiological range of pressure changes because at these low values a scatter of 5–10 mm Hg is seen. (By the method used a pressure can be determined with an error of less than one mm Hg). The observation that proximal intratubular pressures within a single normal kidney in a stable state of function may range between 8–10 and 18–20 mm Hg with a mean pressure of about 13–14 mm Hg (Gottschalk and Mylle 1956 and the personal unpublished observation by the present author) actually indicates that pressure differences may exist between single proximal lumina and the surrounding interstitial and intratubular pressures. Repeated measurements of the intratubular pressure at the same site of puncture in a proximal convolution showed also that it would either remain constant for some minutes at least or vary over a range of several millimeters of mercury although the experimental conditions remained unchanged. Moreover it was demonstrated by registration of the pressure change in proximal tubules and peritubular capillaries following either abrupt clamping of the aorta above the renal arteries or injection of a large dose of a vasoconstrictor substance (angiotensin) that the proximal tubular wall – at least during short term changes in hydrostatic pressures – can resist a moderate positive pressure both from the outside and the inside of the wall (Leyssac 1965 a). By abrupt clamping of the aorta the peritubular capillary pressure dropped to zero almost immediately whereas the proximal intratubular pressure only had reached a value of 6–7 mm Hg in about 3 sec at which time the capillary pressure was zero to decline gradually to zero 12–20 sec after clamping indicating that the tubular wall can resist such positive pressure from the inside for this short time interval. Occasionally the peritubular capillary pressure was found to increase to a peak following the injection of angiotensin whereas the proximal intratubular pressure invariably decreased immediately after similar injection, indicating that the tubular wall also can resist a moderate positive pressure from the outside. Thus the cellular wall of the proximal tubules seems to be characterized by some rigidity sufficient to resist moderate differences of pressure in the lumen and the interstitium. This fact is true at least for short term pressure changes but may possibly be valid also when the changes are

maintained for more prolonged intervals of time (minutes). The physical characteristics of the proximal tubular wall are apparently compatible with the concept that primary changes in the proximal  $T_m$  for salt and water may be responsible for the changes observed in the rate of glomerular filtration within a certain range which is not yet quite defined.

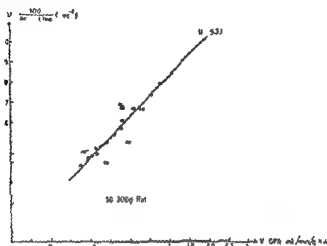


Fig 1 Reciprocal occlusion time ( $U$ ) at spontaneously different clearance of inulin ( $I$ ) in control rats (Leysac 1965 b)

Fig 1 demonstrates the direct proportionality between the clearance of inulin and the reciprocal of the occlusion time (representing the rate of proximal reabsorption) in normal rats. It is seen that the spontaneous variation in the rate of reabsorption (a variation in the reciprocal occlusion time from  $1/11$  to  $1/25$   $\text{sec}^{-1}$ ) corresponds to a variation in the rate of filtration from about  $1.6$   $\text{ml/min/g K.W.}$  to about  $0.8$   $\text{ml/min/g K.W.}$  As it has been shown directly (see Ch. III) that the rate of reabsorption is the independent variable ( $T_m$  limited) some factor(s) must be looked for in order to understand how these occurring changes in the  $T_m$  are effectuated. It was suggested by Boyesen (1954 b) that some humoral factor(s) may be influencing the  $T_m$  because changes in the reabsorptive capacity were seen to be related to changes in the renal plasma flow. Obviously such search would seem meaningless if the reabsorption rate were to change as an automatic consequence of a change in the filtered load.

Goormaghtigh (1940) was the first to attribute an endocrine function to the juxtaglomerular apparatus primarily because of the histological appearance

of the granular, epithelioid cells of the afferent arteriole ( polkissen ) at the very end of that vessel According to Barjas and Latta (1963) and Faarup (1964) the juxtaglomerular apparatus is defined as the afferent and the efferent arterioles containing the epithelioid cells, cells from the adjacent distal tubule (the macula densa), and the Goormaghtigh cells interposed between the macula densa cells the arterioles and the glomerulus In the following discussion the latter definition of the juxtaglomerular apparatus shall be used From serial sections of this anatomical structure from subcapsular and juxta medullary nephrons Barjas and Latta (1963) and Faarup (1964) reconstructed three dimensional models which show that the ascending limb of Henle always approaches the efferent vessel first following it to the vascular pole of the glomerulus here the macula densa cells contact the Goormaghtigh cells and usually the epithelioid cells of the efferent and afferent arterioles The distal tubule (here defined from the macula densa to the collecting duct) will then, after a few convolutions apart from the glomerulus, join and follow for some distance the afferent arteriole before it merges with a distal tubule of another nephron into a collecting duct This peculiar anatomical arrangement characteristic of each mammalian nephron, has naturally puzzled many investigators besides Goormaghtigh who was the first (1940) to suggest on purely morphological evidence that renin - a proteolytic enzyme - is located to the juxtaglomerular cells (epithelioid cells) Later, extensive investigations have firmly established that renin is located and probably formed in the juxtaglomerular apparatus Thus Cook et al (1956) and Bing and Wiberg (1958) showed by direct renin bio assay that the subcapsular aglomerular zone (cortex corticis) did not contain measurable amounts of renin nor did the glomerular capillary tufts removed by microdissection, Cook and Pickering (1959) demonstrated conclusively that only the glomerular fraction contained renin viz by isolating selectively magnetic iron dioxide loaded glomeruli from a suspension of kidney fragments using an electromagnet They also showed that the renin in this fraction was found in the one half of the glomeruli containing the vascular pole Further microdissection studies by Bing and Kazimierzak (1960) confirmed this result but also showed that isolated afferent arterioles contained less than the total juxtaglomerular renin content and that the perivascular cell fraction contained even more renin than did the vessel Well over 50 to 90 per cent of the renin was found actually in the part of the distal tubule containing the macula densa and a smaller fraction less than 50 to 10 per cent was located in the isolated vas afferent which could not be completely separated from the adjacent macular and/or Goormaghtigh cells (Bing and Kazimierzak 1962) These results, indicating directly that the greater part at least of the renin is located in the macula

densa and/or Goormaghtigh cells are apparently contrary to observations by Edelman and Hartroft (1961) who found that fluorescent antibodies to a purified (although not quite pure) renin preparation gave a specific staining of the granular epithelioid cells of the afferent arteriole (JG cells), whereas no staining was seen in the macula cells. Furthermore it has been shown repeatedly (e.g. Tobian 1960) that a correlation is rather fair between the renal renin content and the granularity of the JG cells. Kaplan and Friedman (1942) demonstrated the presence of renin in the meso- and metanephros of the hog foetus lacking epithelioid cells, the finding has been confirmed in recent microdissection studies by Bing and Kazmierczak (1964) using kidneys of new born pigs in which renin was found in the subcapsular zone including premature macula cells but devoid of afferent and efferent vessels. Consequently it can hardly be doubted that renin is located at any rate in the macula densa. According to Bing (1964) it is most likely that renin is formed in the macula densa cells from which it passes to the epithelioid cells of the vessels where it is deposited (possibly as or in the granules). Subsequently it may either pass into the blood circulation as renin or come into reaction with angiotensinogen (an  $\alpha_2$  globulin) from the plasma. In the latter case the product of the enzyme reaction - the decapeptide angiotensin I (Skeggs et al 1957) - might be released directly into the circulation to be immediately converted to the highly potent vasoconstrictive octapeptide angiotensin II (Skeggs, Kahn and Shumway 1956).

The location of the vasoactive renin-angiotensin system specifically to the characteristic structural arrangement of the juxtaglomerular apparatus together with ample evidence of the fact that the renin content is influenced by the salt balance and the action of mineralocorticoid hormones (cf Ch V B) made it rather suggestive that angiotensin in addition to its renal vasoconstrictive action might also be the humoral substance which is responsible for the changes occurring in the proximal salt and water transport. It might be the factor foreseen by Bojesen (1954b, 1955, 1957).

## B THE TUBULAR ACTION OF ANGIOTENSIN

Previous attempts to demonstrate a primary tubular effect of angiotensin were complicated by the invariable and immediate vasoconstrictive effect following the administration of the pressor substance. As appears from the foregoing discussion (p 71-73) such effect could not be revealed by clearance studies. Hence it can hardly surprise that apparently conflicting conclusions have been drawn when such data obtained in studies on different species of

animals as well as in studies on normal as opposed to diseased human subjects were to be interpreted. Nor could it be expected that such effect could be established by direct methods (micropuncture studies) in experiments in which the renal blood circulation was intact (as tried by Ullrich personal communication), since effects, if any, will be accompanied by a significant change in the plasma flow and probably also in the glomerular propulsive pressure, obscuring whether a change observed in the tubular function is a primary or secondary event. Another direct approach was tried by Gertz (1962) who microinjected a saline solution containing angiotensin into the lumina of isolated parts of proximal convolutions and measured the flux of sodium and water (a detailed description of this method has been given in the previous chapter, p. 52). By this method the vascular action of angiotensin could be avoided but no tubular effect was observed at such low concentrations, which may be considered physiological. An inhibition of the net sodium flux of about 30 per cent could not be observed until angiotensin had been introduced into the lumen at a concentration of about 10 mg per 100 ml of solution. This negative result, however, did not exclude that angiotensin might have an effect at physiological concentrations when supplied from the peritubular side of the cells. In analogy, vasopressin also an octapeptide is generally believed to act from the blood side on the water flux and the sodium transport of the distal tubules and collecting ducts by altering the permeability properties of the luminal membrane system. This assumption was adopted after it had been demonstrated by Koefoed-Johnsen and Ussing (1953) that the application of vasopressin (antidiuretic hormone or ADH) to frog skin increases the water permeability of the skin if added to the inside solution while no effect was observed when added to the outside bathing solution (net flux from the outside to the inside of the skin). This effect was located to the outward facing membrane (MacRobbie and Ussing 1961) as indicated by measurements of swelling rates of the hormone treated epithelium when swelling was induced by changing the osmolality of the outside solution. Similarly Hays and Leaf (1962) using toad bladder demonstrated that the increased net flux of water from the mucosal to the serosal side induced by adding vasopressin to the serosal medium was accompanied by an increased concentration of tagged water (THO) in the epithelium provided the tagged water was added to the mucosal bathing solution. This result clearly indicates that vasopressin increases the permeability of a barrier located to the mucosal surface of the bladder. Further the stimulating effect of vasopressin on the sodium transport in the frog skin was shown to be due to a reduction of the partial resistance to the sodium current at some site in the skin whereas the electromotive force of the sodium transporting mechanism remained almost





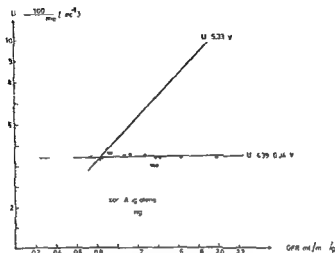


Fig 2 Reciprocal occlusion times after injections of 25 ng of angiotensin at different clearances of inulin measured immediately before the administration of angiotensin (Leyssac 1965 b)

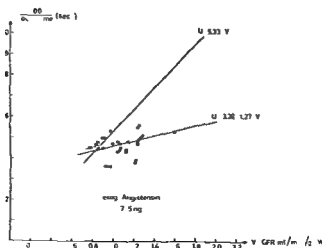


Fig 3 Reciprocal occlusion times after injections of 7.15 ng of angiotensin at different clearances of inulin measured immediately before the administration of angiotensin (Leyssac 1965 b)

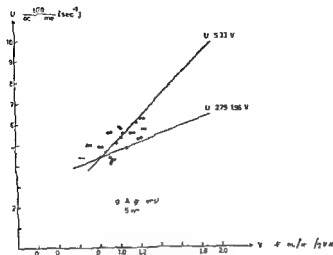


Fig 4 Reciprocal occlusion times after injections of 5 ng of angiotensin III different clearance of inulin measured immediately before the administration of angiotensin (Leyssac 1963 b)

The immediate effect of three doses of synthetic angiotensin II amide on the reciprocal occlusion time ( $u$ ) at spontaneously different clearances of inulin ( $v$ ) is shown in the figures 2-4. An effect is seen to be clearly demonstrated at all three dose levels increasing with the dose administered until a maximum occlusion time (about 23 sec) is approximately reached by the injection of 25 ng of angiotensin. This maximum equals the average maximal occlusion time observed spontaneously (Fig 1) which corresponds to an inulin clearance of about 0.85 ml/min/g KW.

A quantitative description of the observed tubular effect caused by the injected doses of angiotensin ( $d_i$ ) at any preexisting rate of reabsorption which must be determined by the equivalent to or a certain but unknown dose of endogenous angiotensin ( $d_e$ ), is derived from an assumed dose response relationship of the form

$$v(d) = \beta e^{-\gamma d} + \alpha$$

in which  $\alpha$ ,  $\beta$  and  $\gamma$  are positive constants and  $d$  the total dose of angiotensin (for details see Appendix). By means of linear regression analysis approximate values of  $\alpha$  (0.83 ml/min/g KW) and  $\gamma$  (0.2 ng<sup>-1</sup>) were estimated. Using the above dose response relationship the reciprocal of the occlusion times following an injected dose of angiotensin will yield a straight line when plotted as a function of the preexisting clearance of inulin  $v(d_1)$ .

Using the estimated values of ( $\alpha$ ) and ( $\gamma$ ) the observed data are described by the regression lines given in Tabel I and shown in the Figs 2-4. At any pre-existing inulin clearance the effect of the injected dose of angiotensin is indicated by the vertical interval between the regression line ( $u$ ) = 5.33 ( $v$ ), and the regression line calculated on the basis of the occlusion times observed immediately after the administration of angiotensin.

From the above exponential dose response relationship the effect on the occlusion time caused by the three doses of angiotensin as a percentage of the maximally possible effect may be derived (see Appendix) and was calculated to be about 60 per cent, 75 per cent and 99-100 per cent with 5 ng, 7.15 ng and 25 ng respectively.

$v(d_1=d_0)$ \ $v(d_1)$	0.85	0.84
1.00	11	14
1.30	16	19
1.60	18	22

Table II Shows doses of exogenous angiotensin (ng) calculated to reduce  $Cl_{in}$  from three spontaneous levels down to 0.85 and 0.84 ml/min/g KW respectively.

In table II is given the calculated amount of exogenous angiotensin ( $d_+$  expressed in units of  $10^{-6}$  g) necessary to reduce the inulin clearance in rat kidney from three different values ( $v(d_1)$  ml/min/g KW) to 0.85 or 0.84 ml/min/g KW ( $v(d_1 + d_+)$ ) respectively.

Thus the data given in figs 2-4 indicate that under these experimental conditions the maximal effect brought about by exogenous angiotensin is a primary inhibition of the proximal reabsorption rate to a minimum value which in rats gives a glomerular filtration rate of about 0.8 ml/min/g KW, this is equal to the lowest spontaneous value observed. This immediate and direct effect was shown to be rapidly reversible (Leyssac 1964 a). A similar direct inhibitory effect of angiotensin on the proximal reabsorption rate was further demonstrated by repeated registrations of the proximal intratubular pressures during the pressure decline induced either by aortic clamping or by intravenous administration of 25 ng of angiotensin (Leyssac 1965 a). The intra

tubular pressure was found to reach zero value within 12-20 sec following complete aortic clamping at which time the lumina were completely emptied of fluid. When 25 ng of angiotensin are injected into a rat the vasoconstrictive effect acting also on the afferent vessel (if applied into the systemic circulation) will abolish for a moment more or less completely, the renal circulation (as indicated by the cessation of the red cell circulation in the peritubular capillaries), thus imitating the effect of aortic clamping consequently the glomerular filtration will cease. The proximal intraluminal pressure decline following such injection aimed towards zero value in about 20-30 sec as opposed to 12-20 sec seen when no angiotensin was administered the zero value was reached only occasionally because of the very brief vascular effect which generally would allow filtration to be reestablished before the proximal lumina were completely emptied. The more slow pressure decline observed in the presence of angiotensin may be taken as a further direct evidence that the rate of proximal fluid reabsorption from the lumen is decreased on account of a primary tubular action.

Finally a direct tubular action of angiotensin was also suggested by Langford and Fallis (1963) on the basis of experiments with the Sperber chicken preparation. A more detailed report on these experiments was given by Langford (1964). It was shown that infusion of 0.1  $\gamma$ /kg/min or more of angiotensin via the leg vein to the renal portal circulation of one of the kidneys induced a promptly increased urine flow and sodium excretion rate ipsilaterally. Also infusions of noradrenaline (5  $\gamma$ /kg/min) provoked a prompt diuresis but there was no lateralization and the urinary sodium concentration decreased with increasing urine flow after noradrenaline as opposed to an increased sodium concentration with diuresis following infusions of angiotensin. Glomerular filtration rates as estimated by inulin clearance in 5 hens were somewhat unstable and no significant effect of angiotensin could be demonstrated. These results suggesting a direct tubular inhibition of sodium and water reabsorption in the avian kidney fail to reveal to which tubular section the action might be localized and may be subject to the objection that a vascular action of angiotensin on the afferent and/or efferent arterioles is not excluded by the Sperber technique since these vessels also are surrounded by the peritubular capillaries draining from the renal portal system.

A dose of 15-20 ng of angiotensin might at first glance appear to be a rather heavy (unphysiological) dose which could elevate the systemic arterial pressure considerably. It should be borne in mind however that the tubular effect is measured only 9 sec after start of the injection at which time only a minor fraction of the injected amount of substance has actually reached the kidney. By simultaneous injection of similar doses of pressor substances

with  $^{131}\text{I}$  labelled albumin and removal of the kidney 9 sec after start of the injection, it was indicated that only a fraction of 2-4 per cent of the injected amount of albumin had reached the kidney in that time (Leyssac 1965 b). Assuming that also as little as 3 per cent of the injected amount of angiotensin is present in the kidney at the instant of registration of its effect a maximum effect is obtained when about 0.7 ng of exogenous angiotensin is acting per g of rat kidney equal to about 1.2 ng (or  $1.2 \times 10^{-12}$  mol or  $7 \times 10^{11}$  molecules) per g of tubular cells (which corresponds to about 60 per cent of the kidney's volume). If the spontaneous variation in the rate of proximal reabsorption of fluid (or salt and water  $T_m$ ) is further assumed to be elicited by various concentrations of endogenous angiotensin the data indicate that a difference in the amount of endogenous angiotensin in a rat kidney corresponding to about  $7 \times 10^{11}$  molecules per g of tubular cells may be responsible for the physiological interval of variation in the  $T_m$ . The proximal tubules are highly sensitive to a change of this order of magnitude. Therefore the amount of free endogenous angiotensin in the kidney must probably be of the same order of magnitude. With cubic cells of a thickness of 6-7 microns ( $\mu$ ), a change in the order of 200 molecules per cell will be sufficient to elicit a maximum physiological depression of the proximal reabsorption rate, apparently a reasonably low number for a physiologically active hormone. The localization of the renin-angiotensin system to the juxtaglomerular apparatus and the demonstrated rapidly reversible and momentary tubular action of angiotensin with its characteristic maximum strongly suggests that angiotensin also exerts its vasoconstrictive action in the same area which readily might explain the whole physiological range of variation in the renal blood flow as well as in the proximal  $T_m$  for salt and water and rate of filtration. *On this basis it may be justified to conclude that the renin-angiotensin system is a physiological regulator of these parameters.*

The observation of a characteristic maximum in the tubular inhibitory effect of injected angiotensin represents the greatest challenge to the comprehension also of the integrated function of the kidney. If a similar maximum is also characteristic for endogenously released angiotensin even an abnormal increase in the release should not be able to depress the rate of filtration below the physiological range of variation (i.e. below a value of about 0.8 ml/min/g K.W. in rats) by its action on the proximal tubular transfer of fluid. Other - supplementary - factors or mechanisms has to be postulated to explain such further reductions in the mulin clearance observed under pathological as well as under various experimental conditions. By application of rather tight Goldblatt silver clamps to renal arteries of rats in acute experiments by which the arteries were partially occluded to different degrees depending on the

size of the rat examined, it was demonstrated as mentioned in the preceding chapter (p 66-67) that the reciprocal of the occlusion time had reached a fixed minimum level almost identical to that obtained by injections of 25 ng of synthetic angiotensin and equal to the lowest value seen spontaneously (Leysac 1964a Fig 2) The data obtained under this condition of partial arterial clamping indicate directly that the depression of the proximal reabsorptive capacity only can account for a depression of the turnover rate to a value of about 0.8 ml/min/g KW. The measured clearances of inulin were reduced far below this value (occasionally down to below 0.1 ml/min/g KW). Other mechanisms therefore had to be looked for. It was observed in the same study that the urine flow decreased in direct proportion to the abnormal reduction in inulin clearances below 0.8 ml/min/g KW in contrast to the fact that such correlation was non-existing within the physiological interval of variation in accordance with the general experience. Moreover, since urine osmolality decreased - approaching isosmolality - *pari passu* with decreasing urine flow in this low and abnormal interval the observation suggested that the further reduction in clearances of inulin under these conditions was caused by proximal luminal occlusion of more and more nephrons. Such luminal occlusion of a varying number of nephrons was directly demonstrated in kidneys frozen at states of function characterized by clearance values below 0.8 ml/min/g KW in contrast to wide open lumina of all nephrons in kidneys frozen at a physiological state of function ( $Cl_{in} \geq 0.8$  ml/min/g KW). These results might suggest indirectly that endogenous angiotensin also exhibits a characteristic maximum in its tubular action and that a depression of the proximal reabsorptive capacity due to angiotensin cannot exceed that observed in the clipped kidneys even if the release of angiotensin is abnormally increased in such kidneys as may be assumed although it has not yet been conclusively proven.

A final conclusive demonstration that the release of angiotensin actually varies in inverse proportion to the proximal  $T_m$  for salt and water and renal blood circulation within the physiological interval of variation and that the release is abnormally increased in partially stenosed kidneys would definitely confirm the above conclusions. It therefore should be considered an important object for future studies to elaborate a sufficiently sensitive analysis for angiotensin permitting a quantitative determination of such minute variations as are prone to occur physiologically in the renal capillary or venous blood. Until this has been achieved the most reasonable concept which satisfactorily may explain the available observations, seems to be that physiological variations in the renal blood flow and the proximal reabsorption rate (and thus in the glomerular filtration rate) are mediated by changes in the release of angio-

tensin. Further (abnormal) reductions in the renal blood flow and filtration rate are due to lowered arterial and glomerular propulsive pressures whereby the number of nephrons participating in urine formation gradually is reduced. In view of this concept it may be predicted that the glucose  $T_m$  ( $T_{mG}$ ), as inferred from clearance studies, apparently would be decreased at such low pressures. In accordance herewith this was also observed by Kruhoffer (1950) using dehydrated rabbits, in which  $T_{mG}$  changed in parallel with clearances of inulin below values in the latter of about 10 ml/min, whereas no change in  $T_{mG}$  was observed above this level. The term "apparent decrease in  $T_{mG}$ " has been used here because the calculated reduction in the amount of glucose reabsorbed per unit of time in accordance with Kruhoffer's own interpretation is taken as an indication that only the number of active nephrons was reduced by occlusion without necessarily implying that the cellular capacity of the transporting mechanism had changed.

The disclosure of the two primary renal effects of angiotensin, viz the vasoconstrictive and the tubular effects, satisfactorily explain the established interrelationship of renal blood flow, proximal reabsorption rate, and glomerular filtration rate under physiological variations as due to the regulation by one common factor, and opens the interesting question with which parameter the regulation is primarily concerned.

## CHAPTER V

### A A REGULATED PARAMETER

On the basis of the foregoing discussion it may be accepted that physiological changes in the renal blood circulation and in the functional states of the proximal segment of mammalian nephrons, estimated by the rates of proximal fluid reabsorption (or turnover rates) of the integrated nephron population are mainly at least reflections of a regulated release of angiotensin from the juxtaglomerular apparatus of the individual nephrons. However these investigations have failed to provide an answer to the question whether and how, the juxtaglomerular apparatus may serve as a link by which the functional states of the proximal and distal segments become mutually interrelated.

The absolute necessity of a functionally adjusting link is obvious from the Tm limited transfer rate also of the fraction (80 %) of the filtered volume which is reabsorbed proximally. The problem to be faced appears from the general biological requirement of salt balance which in mammals is maintained exclusively by a regulated renal salt excretion. The renal salt excretion rate must therefore be quite *uninfluenced* by factors unrelated to the salt balance factors which primarily may change either the tubular reabsorption rate (metabolic changes) or the rate of filtration of salt (changes in the glomerular propulsive pressure brought about mainly by changes in preglomerular vascular resistance).

How this delicately adjusted salt excretion rate may be brought about in the mammalian kidney in spite of these disturbing influences can hardly be understood on the basis of discussions of conditions for a regulation of salt excretion by the integrated kidney since the integrated renal function involves too many unsolved and highly complex problems because of the anatomical and probably also functional inequality of the nephron population in kidneys of various species especially regarding the cortical and juxtamedullary nephrons and their unanalysed relative participation in the excretion rate of salt.

Consequently the discussion should be restricted to include considerations on the conditions necessary for a regulated salt excretion rate by the single nephron. In this context the suggestion of a small and limited distal reabsorp-



tive capacity of sodium, as inferred from the study by Wesson, Anslow and Smith (1948) on osmotic diuresis (cf Ch I, p 20) seems to be of relevance, and has been confirmed also in recent micropuncture studies by Giebisch, Klose and Windhager (1964). These authors showed that the fraction of filtered load of sodium reabsorbed along the distal tubule, as indicated by the F/P sodium divided by the F/P inulin plotted against the sites of collection in this segment was greatly reduced in hypertonic saline loaded rats as compared with conditions in normal control animals, calculations on the basis of the simultaneous percentile increments in plasma sodium concentration and filtration rates showed that the absolute rates of reabsorption in the distal tubules either had increased to a minor degree remained unchanged, or frequently had slightly reduced under these loading conditions in which the sodium and water excretion rates were markedly elevated. Furthermore it is generally agreed now that mineralocorticoid hormones play a dominant role in the physiological regulation of salt excretion by enhancing the distal transfer processes particularly those responsible for an exchange of sodium with other cations. This assumption of a distally located site of action is generally substantiated by stop flow experiments by Vander et al (1958) and Vander Wilde and Malvin (1960), who demonstrated that the level of the sodium concentration in the early samples representing fluid from the distal segments is much higher in normal control dogs than in adrenalectomized animals on administration of aldosterone this distal tubular defect may become normalized. But as this effect of mineralocorticoid hormones on the sodium excretion rate is considerably delayed (45-60 min), as repeatedly demonstrated, first on extracts of whole adrenal cortex (e.g. Roemmelt, Sartorius and Pitts 1949), and as the distal transfer capacity for sodium ( $Tm^d_N$ ) probably is relatively small and limited it follows that the significance of aldosterone as a regulator of salt excretion must be conditioned by a fairly constant load of sodium to the distal part of the nephrons. Finally as the volume of fluid supplied to the distal tubules is determined by the difference of the hydrostatic pressure at the end of the proximal segment and the beginning of the distal tubule and also by the resistance to flow across the loop of Henle which resistance can hardly be actively changed (absence of muscular cells) it must be presumed that the proximal intratubular pressure has to be kept fairly stable by some intrarenal regulating mechanism. In accordance with this argumentation it should be mentioned that Gottschalk and Mylle (1956) showed that the mean proximal intratubular pressure of 13.5 mm Hg (119 recordings in 56 rat kidneys) varied within a range of 7 to 21 mm Hg with a standard deviation of only 2.4 mm Hg and that the average pressure in the proximal lumina in each rat was the same ( $13.3 \pm 3$  mm Hg). Similar values were

obtained in control kidneys under similar experimental conditions (Leyssac 1964 b) besides pressures in single proximal convolutions of one kidney were seen to vary within a similar range of about 9 to 20 mm Hg from one area of the surface to the other at any particular time. Such fairly relative constancy of the mean proximal intratubular pressure from one kidney to the other and in the individual kidneys at different times within a pressure range, in which the tubules have more or less the character of rigid tubes (cf preceding chapter p 73-75), might suggest that the intratubular pressure in the single nephron actually is controlled or stabilized by a regulating mechanism. Experimental evidence supporting this suggestion was presented also by Leyssac (1964 b), who showed in acute experiments that the application of a rather tight partial constriction (Goldblatt clamp) of the renal artery which reduced the inulin clearance to values far below the normal range ( $<0.8$  ml/min/g K.W.) did not influence to measurable degrees the proximal intratubular or peritubular capillary pressures whereas the distal intratubular pressure was seen to be reduced significantly. Direct evidence had been presented (cf p 85) that the abnormally reduced inulin clearance in such kidneys is due to a cessation of the luminal fluid flow and subsequent luminal occlusion of the proximal convolutions attributable to a more or less complete proximal reabsorption of the filtered fluid provoked by the reduction in the glomerular propulsive pressure following partial arterial clamping in a state of fixed proximal Tm for salt and water. Thus the glomerular capillary hydrostatic pressure in these kidneys must have been significantly lower than normal. The proximal luminal pressure of still functioning nephrons remained unchanged. It therefore seems safe to conclude that the proximal intratubular pressure is subject to a very efficient regulating mechanism and that the fair constancy of the mean proximal intratubular pressure from time to time and from one normal rat to the other is a reflection of this regulation. Since further it may be assumed that these normal (control) rat kidneys have represented the greater part of the entire range of spontaneous variations in the salt turnover rate and blood flow it might be tempting to relate also the relative constancy of pressure to these variations in other words tentatively to suggest (or infer) that the physiological changes in the release of angiotensin by its vascular and tubular effects were responsible for the regulation of the proximal intratubular pressure.

It may be questioned whether it is essential to know exactly the magnitude of the pressure decrement across the glomerular membrane in order to accept that the tubular action of angiotensin plays a significant part in the regulation of the proximal intratubular pressure. But such knowledge is hardly of any importance as may emerge from the following argumentation. Thus 1) if there

is only a moderate resistance to flow across the membrane a greater rate of filtration must be conditioned by greater pressure decline (i.e. greater effective filtration pressure). If this resistance is inferior to the resistance offered by the distal segments of the nephron (viz from the beginning of the loop of Henle to the renal pelvis), in conformity with previous conclusions (p 70-71), any change in filtration rate i.e. in effective filtration pressure must be determined by changes in the rate of proximal reabsorption ( $Tm^*_N$ ) which primarily changes the intratubular pressure. Conversely changes in the glomerular propulsive pressure will involve primarily an almost parallel change in the proximal, intratubular pressure but no perceptible change in the filtration rate (cf p 71). Realizing quite well that it has never been verified in experiments it might serve as a hypothetical example that the extremes of the normally occurring variations in the proximal intratubular pressure (7-19 mm Hg) might represent extremes of an 'incompensated' state, viz variations of about 6 mm Hg to each side of the compensated mean pressure of 13 mm Hg. This variation would also represent the *maximum* change in the effective filtration pressure, since regulated changes in the reabsorption rate induced by endogenous angiotensin normally will be accompanied in all probability, by changes in the tone of the efferent arteriole (cf discussion below, p 104-105) and thereby in the glomerular capillary hydrostatic pressure which will change in the same direction as the intratubular pressure change elicited by the changed reabsorption rate. Consequently in rats a change in the filtration rate from 0.8 to 1.6 ml/min/g K.W (normal range of variation) should correspond to a change in the effective filtration pressure not exceeding 6 mm Hg. On the assumption that the rate of filtration is linearly dependent on the effective filtration pressure 0.8 ml/min/g K.W becomes filtered at an effective filtration pressure of 6 mm Hg 1.6 ml/min/g K.W being filtered at a pressure of 12 mm Hg i.e. declines in the pressure across the glomerular membrane ranging at levels not exceeding 6 to 12 mm Hg determined by the rates of proximal reabsorption.

2) If on the other hand the pressure drop across the glomerular membrane is considered to be small as compared with the oncotic pressure (but still with a proximal transfer of salt of  $Tm$ -character), an increase in the glomerular propulsive pressure would still be accompanied by an almost parallel increase in the proximal luminal pressure and a proportional increase in the rate of excretion of salt and water. A pressure regulation by which the proximal reabsorption rate is *not* simultaneously changed i.e. mediated solely by an efferent arteriolar relaxation would then involve an increased renal blood flow and since the filtration rate had not changed significantly, a reduction in the filtration fraction ( $C_{FR}/RPF = FF$ ). Consequently the final

glomerular oncotic pressure will be below the normal level and the glomerular propulsive pressure and proximal intratubular pressure will remain elevated in spite of a normalized capillary hydrostatic pressure. These two effects of an efferent arteriolar relaxation apparently will counteract each other and changes in the oncotic pressure become highly significant to the intratubular pressure. Thus, in order to maintain the proximal luminal pressure unchanged the glomerular hydrostatic pressure should overcompensate exactly to a degree at which it counteracts the change in oncotic pressure. This will be found to be a highly vulnerable regulating mechanism, since it has to rely solely on the control of the resistance offered by the one efferent arteriolar vessel. In addition the vascular resistance to flow being determined by the internal radius in the fourth power such intratubular pressure regulation would require an extremely delicate and exact adjustment of the arteriolar tone. This regulation would be far less critical if the tubular reabsorption also were to play a significant part in the pressure regulation. However, angiotensin is found by now to have actually a tubular action, by which the filtration rate does change in such a way that changes in the filtration fraction are avoided. The inference hereof is that the tubular action also in this case has a stabilizing (or regulating) effect on the proximal intratubular pressure.

## B CONDITIONS OF CHANGED RELEASE OF ANGIOTENSIN OR RENIN

The question to emerge next is whether or not the above hypothetical concept of changes in the release of angiotensin being brought about in order to regulate the proximal intratubular pressure is compatible at all with the results obtained from investigations on the adequate stimulus causing a release of angiotensin or renin. It has been shown unequivocally in several laboratories that angiotensin (in the sense of the octapeptide angiotensin II) is the active substance but the analytical methods remain to be perfected to a degree of sensitivity at which it might be established whether the juxta glomerular apparatus under physiological conditions releases angiotensin and/or renin directly into the blood circulation in which angiotensin may be formed instantaneously by renin reaction with the plasma substrate (angiotensinogen). The presence of renin also in normal human and rabbit plasma as indicated by recent investigations reported by Peart's group in London (Brown et al 1963 b, Lever and Tree 1963) who used the best and for the time being most sensitive renin assay strongly suggests a direct release of

renin into the blood. Whether this is true also for angiotensin remains to be established. But the degree of correlation is fair, apparently, of changes in plasma concentrations of renin and angiotensin under a variety of abnormal conditions and of such changes in the plasma concentration and in the renal renin content, hence it seems to be justified to discuss collectively these studies.

a) *Renal renin content in kidneys in which the release of renin and/or angiotensin assumedly is increased*

Numerous studies of the renin content in kidneys and plasma have been published since the time when Goldblatt and collaborators (1934) demonstrated in dogs that arterial hypertension might be provoked by partial clamping of the renal arteries. In brief the renin content has been estimated by several types of bio assay either from crude or from more or less purified saline extracts of kidneys either directly, as renin, or by the indirect test, by which renin is incubated with substrate under standard conditions and the yield of formed angiotensin is estimated. It is outside the scope of this review to enter into a detailed discussion about the many objections raised to the different types of assay preparations, only it shall be mentioned that by now it seems generally accepted that intravenous injection of the test solution into a normal or nephrectomized rat or other mammal and subsequent registration of the increase in the blood pressure (dog test or rat test) is the bio-assay which is most reliable and hence the one of choice. It should be briefly mentioned also that the only reliable results to be obtained by the indirect renin test (estimating the formed amount of angiotensin) originate in studies in which the inactivation of angiotensinase prior to incubation may be regarded as complete as emphasized by several authors (e.g. Kemp 1962). Results obtained by the direct test in which crude extracts are injected should be taken also with a certain reservation because the presence of depressor material may represent a severe source of error. An additional although less likely source of error involved in this method might be a difference in the renal content of angiotensinase indications of which were seen by Bing (1962 a) in clamped kidneys versus control kidneys. The formation and pressor action of angiotensin being extremely rapid however this error will probably be insignificant. In view of these many sources of error it can hardly surprise that a rather complex and often contradictory picture is obtained from the earlier literature and consequently a few only of the most thorough investigations using the most reliable methods for the identification of the pressor substance either as renin or

angiotensin shall be included here any further comments on the particular procedures of identification are not given

Pickering Prinzmetal and Keisall (1942) used the direct renin test of an extract of rabbit kidneys and found that the renin content had increased as compared with normal in kidneys during the first week after the application of a partial clamp to the renal artery. In rabbits in which chronic hypertension (Goldblatt hypertension) persisted for 2-6 months the renal renin content was normal. Similarly Haas and Goldblatt (1959) showed in dogs that the renin content was about 2-8 times greater in the clipped kidney than in normal kidneys 13-30 days after application of the clamp and further, that the renin content was reduced in the untouched, contralateral kidney. Also in hypertensive patients they found an increased renal renin content as compared with normal human kidneys. Unfortunately it is not reported whether or not these patients were renal hypertensives nor whether the hypertension was of a benign or malignant nature. The conclusion drawn from these results has been further established by Bung (1962 b) in experiments on rats in which one renal artery was partially clamped and is in general accordance with results obtained in several other investigations. Further it has been substantiated by observations of parallel changes in the number and intensity of staining of the granules of the juxtaglomerular cells (the juxtaglomerular index or JGI) since it has been reported by Tobian, Janecsek and Tomboulain (1959) that the JGI as well as the pressor substance activity was increased in the partially clamped kidney but decreased in the contralateral untouched kidney. An increased pressor activity most likely being due to renin was observed also in adrenalectomized rats by Gross and Sulser (1957) whereas pressor activity and JGI were reduced in kidneys of rats treated with desoxy corticosterone acetate (DOCA) and salt (Tobian, Janecsek and Tomboulain 1959) or according to Tobian (1960) by heavy peroral salt loading alone. It should be emphasized however that although later investigations to be referred below strongly have suggested that an increased release of renin and/or angiotensin is not infrequent in such clamped kidneys in which the renin content and/or concentration have increased it has not been established whether this is *always* the case and the mere finding of a change in the renal renin content makes it hardly justified to infer that a parallel change in the release has also occurred. This fact was further emphasized in the discussions in Quebec where Hartroft (Hartroft, Sutherland and Hartroft 1964) declared that degranulation of the juxtaglomerular cells may occur as an acute phenomenon following immediately upon sodium depletion the latter always being followed by increased granularity provided the sodium depletion is maintained. Consequently a greater significance should be attached to findings

recently obtained in studies on the renin and angiotensin concentrations in lymph and plasma the experiments were carried out under varying conditions and, the methods by which the findings were obtained may be characterized as reasonably safe and reliable. These results have directly confirmed the assumption of an increased renin release under conditions of bleeding shock and within the first weeks after partial constriction of the renal artery, and have gained a further indirect support by the demonstration that other enzymes also may be released from the kidney under such conditions. Kemp (1960) has presented evidence that lactic acid dehydrogenase is liberated to the renal venous blood of the clamped kidney in the early phase of Goldblatt hypertension in rabbits and Kemp and Laursen (1962) demonstrated in cats an increase in serum lactic acid dehydrogenase (LD) - and glutamic oxalacetic acid transaminase (GOT) activity during severe bleeding hypotension. In a few cases they showed by electrophoresis that some of the LD activity could be ascribed to renal isoenzymes of LD.

#### b) *Changes in lymph and plasma concentrations of renin and angiotensin*

Even before the renin assay introduced by Peart's group had been perfected to a sensitivity at which renin could be demonstrated in the renal venous blood, Lever and Peart (1961) had been able to demonstrate an increase in pressor material in lymph collected from the thoracic duct of dogs in which the renal artery was partially constricted. The pressor substance was identified as renin.

A similar increase in renin concentration in thoracic duct lymph has recently been reported by Higgins, Davis and Urquhart (1964) using dogs with experimental, secondary aldosteronism and ascites provoked by thoracic cava inferior constriction. After bilateral nephrectomy the pressor activity was reduced in such dogs.

Similarly Davis and his group (Higgins, Davis, Urquhart and Olichney 1964) reported an increase in the renin concentration in plasma of anaesthetized as well as conscious dogs with thoracic cava inferior constriction. Concentrations of renin were considerably higher in the renal venous plasma than in the peripheral plasma and about 25 times higher than in blood drawn from normal dogs. Further following nephrectomy the activity in the peripheral plasma was seen to decline in all cases. Finally they presented evidence that an excess of plasma angiotensinogen was present in equal amounts both in normal control and experimental animals and consequently it could not represent the limiting factor to the formation of angiotensin in their indirect assay.

A series of investigations carried out under several conditions and concerned with the concentrations of renin and angiotensin in plasma were reported at the international symposium on angiotensin sodium and hypertension held in Quebec October 1963, a few of these investigations shall be referred Scornic and Paladini (1964) assayed preformed angiotensin in arterial plasma of dogs and found significantly increased plasma concentrations after severe bleeding (arterial pressures of 80 mm Hg and below), in nephrectomized dogs bleeding provoked no such effect by which the early observation by Huidobro and Braun Menendez (1942) is confirmed These authors found also an increase in the plasma concentration of angiotensin in acute Goldblatt hypertension after tight partial clamping (in chronically hypertensive dogs such increase was not seen) in salt depleted animals and after infusion of 2-4  $\mu\text{g}$  of noradrenaline per kg per min, by which the systemic arterial pressure was elevated by 20-40 mm Hg However, such increase could not be demonstrated after aortic clamping above the renal arteries by which the femoral arterial pressure was reduced to between 60 and 35 mm Hg whereas a combination of the aortic constriction and noradrenaline infusion (2-4  $\mu\text{g}/\text{kg}/\text{min}$ ) was seen to elevate the peripheral angiotensin level This apparent failure to increase the plasma angiotensin concentration after aortic constriction alone (indicated by their method) as opposed to conditions of bleeding and noradrenaline infusions might be due in part to the fact that these kidneys were not denervated A compensatory, arteriolar vasodilation in response to aortic constriction was not excluded the consequence of which might have been that the pressure at the end of the afferent arteriole or the glomerular propulsive pressure had not reduced to nearly the same extent as during bleeding shock and noradrenaline administration Unfortunately renal blood flow inulin clearance and sodium excretion rate were not estimated in these experiments Their negative finding under conditions of aortic constriction is in contrast to findings by other investigators (e.g. Skinner McCubbin and Page 1963 cf p 97) who found levels of angiotensin like precursor material to be increased in the renal venous plasma only few minutes after reduction of the mean perfusion pressure to the kidney In analogy Vander and Miller (1964) observed increased levels of renin in the renal venous plasma after similar constrictions of the aorta above the renal arteries Their study shall be discussed later in greater detail The reason for the discrepancy between these results seems uncertain The kidneys were not denervated either in any of these latter investigations but it should be mentioned that Scornic and Paladini only tested precursor substance in the peripheral plasma not in renal venous plasma as was the case in the other studies



recently obtained in studies on the renin and angiotensin concentrations in lymph and plasma, the experiments were carried out under varying conditions and the methods by which the findings were obtained may be characterized as reasonably safe and reliable. These results have directly confirmed the assumption of an increased renin release under conditions of bleeding shock and within the first weeks after partial constriction of the renal artery and have gained a further indirect support by the demonstration that other enzymes also may be released from the kidney under such conditions. Kemp (1960) has presented evidence that lactic acid dehydrogenase is liberated to the renal venous blood of the clamped kidney in the early phase of Goldblatt hypertension in rabbits and Kemp and Laursen (1962) demonstrated in cats an increase in serum lactic acid dehydrogenase (LD) - and glutamic oxalacetic acid transaminase (GOT) activity during severe bleeding hypotension. In a few cases they showed by electrophoresis that some of the LD activity could be ascribed to renal isoenzymes of LD.

#### b) *Changes in lymph and plasma concentrations of renin and angiotensin*

Even before the renin assay introduced by Peart's group had been perfected to a sensitivity at which renin could be demonstrated in the renal venous blood, Lever and Peart (1961) had been able to demonstrate an increase in pressor material in lymph collected from the thoracic duct of dogs in which the renal artery was partially constricted. The pressor substance was identified as renin.

A similar increase in renin concentration in thoracic duct lymph has recently been reported by Higgins, Davis and Urquhart (1964) using dogs with experimental secondary aldosteronism and ascites, provoked by thoracic cava inferior constriction. After bilateral nephrectomy the pressor activity was reduced in such dogs.

Similarly, Davis and his group (Higgins, Davis, Urquhart and Olichney, 1964) reported an increase in the renin concentration in plasma of anaesthetized as well as conscious dogs with thoracic cava inferior constriction. Concentrations of renin were considerably higher in the renal venous plasma than in the peripheral plasma and about 2.5 times higher than in blood drawn from normal dogs. Further following nephrectomy the activity in the peripheral plasma was seen to decline in all cases. Finally they presented evidence that an excess of plasma angiotensinogen was present in equal amounts both in normal control and experimental animals and consequently it could not represent the limiting factor to the formation of angiotensin in their indirect assay.

centrations in plasma of these two substances under a variety of abnormal conditions the oversimplification may be accepted at present for the sake of conceptual approach that the release of renin and/or angiotensin under these experimental and pathological states may be considered as caricatured illustrations of a physiological regulating mechanism in which angiotensin is the active factor. In order to simplify the following discussion an increased release of renin and/or angiotensin will be considered equal to an increased release of angiotensin.

It is apparent from the literature *that the release of angiotensin is stimulated* under conditions of salt depletion Addison's disease cardiac insufficiency cirrhosis and other states involving oedema in secondary experimental aldosteronism due to thoracic cava inferior constriction bleeding shock administration of pressure substances (nor adrenaline), and not infrequently after partial constriction of the renal artery or the aorta above the renal arteries in the latter cases probably depending on the degree of arterial obstruction *altogether conditions which have in common a tendency to or a reduction in the glomerular propulsive pressure*. This has been further established by Skinner, McCubbin and Page (1963) by partial obstruction of the renal circulation. They observed an increase in the renal venous plasma of a pressor substance, most likely being angiotensin only 5 min after minor reductions in the mean arterial pressure. Obstructions giving identical reductions in pulse pressure and renal plasma flow but slighter changes in the mean arterial pressure failed to cause any detectable increase in the release of the pressor substance.

Before entering into the discussion whether and how these observations fit with the present concept of angiotensin as a regulator of the proximal intratubular pressure a brief comment shall be made concerning some of the previous hypotheses on the adequate stimulus to the juxtaglomerular apparatus and its possible physiological significance to the renal function.

### C HYPOTHESES ON THE STIMULUS TO ANGIOTENSIN RELEASE AND/OR THE FUNCTION OF THE JUXTAGLOMERULAR APPARATUS

Only a few of the previously proposed hypotheses shall be discussed here the intention being to present only representatives of current ideas on the function of the juxtaglomerular apparatus and the adequate stimulus(1) to release of angiotensin. This topic should not be discussed however, without

mentioning that several anatomists and physiologists have considered and still believe that any physiological function cannot be attributed to this structure which is believed to be a degenerated or passively (mechanically) modified, group of cells

Since Goormaghtigh (1940) suggested that renin might be secreted from these cells several investigators have contributed to our present knowledge of the structural and ultrastructural characteristics of the different types of cells of the juxtaglomerular apparatus and on purely morphological evidence have proposed theories as regards the possible stimulus to the renin-angiotensin system. Thus McManus (1947) emphasized that while the Golgi apparatus of all other cells of the nephron is localized to the luminal side of the nucleus it is placed in the basal side of the macula densa cells and, thus, close to the other constituents of the juxtaglomerular apparatus. This localization of the Golgi apparatus was supposed to be indicative of a secretory activity towards the other cells of the structure (cf Bing's concept mentioned p. 77). Furthermore, McManus pointed out that the basement membrane of the macula densa cells is extremely thin or even interrupted towards the network of Goormaghtigh cells, suggesting that fluid absorbed by the macula cells of the distal tubule easily and without restriction might diffuse between the different parts of the juxtaglomerular apparatus. In accordance with this, Oberling and Hatt (1960), confirmed by electronmicrography, the discontinuity of the basement membrane of the macula densa cells through which membrane these cells are in intimate contact with the lumen or cytoplasmic network of the adjacent cells, suggesting that the composition of the distal tubular fluid in some way might influence the release or production of renin. The same suggestion, viz. that distal fluid osmolar or sodium concentration via the macula densa cells is an important signal to regulation in some way of urine formation, was also advocated by Latta and Maunsbach (1962) who in a study by electron microscopy pointed out the existence of extracellular compartments (vacuoles) at the base of these tubular cells as morphological evidence suggesting fluid transport from the tubular lumen to the other cells of the juxtaglomerular apparatus. Also the occasional observations of an apical swelling of the macular cells were taken as evidence of their osmotic sensitivity. Obviously, other interpretations are possible. Although these structural observations are insufficient and at least fail to suggest how a possible change in the distal fluid composition might influence the angiotensin release or renin production and how this again might influence renal function and sodium balance, they do stress the obvious relevance of the anatomical interrelation of the distal tubule, the arterioles and the Goormaghtigh cells.

In the preceding section (B) a report is given of the evidence provided

that the release of angiotensin is stimulated under conditions of negative sodium balance and other conditions which in this respect imitate a decreased extracellular fluid volume or distension of the vascular system on the basis of the ample evidence collected in support hereof Tobian (1960) suggested that a decreased stretch of the juxtaglomerular cells of the afferent arteriole is actually the stimulus to the renin-angiotensin system. But whether or not such release were of significance to the renal functional parameters was not explained except that it might exert some influence on the adrenal mineralocorticoid secretion thus causing sodium retention by which the extracellular volume would be restored. Tobian's hypothesis is said to be supported by the finding in normal rabbits viz that the juxtaglomerular zone of glomeruli situated near the origin of an interlobular artery contains little or no renin while that of glomeruli closer to the capsule of the kidneys were rich in renin (Brown et al 1963 a) suggesting that this distribution of renin content might be related to the arteriolar pressure, if variations in the renin content indicate parallel variations in renin secretion (Brown et al 1964 b). However their results indicate merely that the renin release may be stimulated under conditions of low arteriolar pressure, but fail to present evidence of whether the stimulus actually is an arteriolar stretch or some other parameter such as the glomerular propulsive pressure or some function thereof which under these conditions will be similarly changed. Hence the inference that such data are in support of Tobian's theory as such is rather unjustified. Schmid (1962) extended the main idea that arteriolar stretch or pressure might be a stimulus to the release of angiotensin. He advanced the theory of a balance mechanism operating across the glomerular tufts between changes in the afferent arteriolar pressure at the site of the juxtaglomerular cells and changes in the post glomerular resistance elicited by angiotensin. The juxtaglomerular cells were assumed to be sensitive to pressure and to increase their release of angiotensin at reduced pressure at this site. An increased efferent resistance as a consequence of the release of angiotensin would thereby increase the intraglomerular pressure. Further the glomerular capillaries were presumed to constitute the return loop of a feed back mechanism to the pressure sensitive juxtaglomerular cells. In effect this mechanism would serve to maintain a constant filtration pressure according to Schmid (cf discussion p 89-91). These ideas of a decreased stretch or pressure and modifications thereof as an adequate stimulus (e.g. suggestions of expansile pulsation or dynamic compliance as proposed most recently by Lowe (1964)) disregards the highly suggestive importance of the junction between the distal tubule and the other constituents of the juxtaglomerular apparatus and seem to disagree with observations by other authors.

In this context attention should be directed to the interesting study reported by Sellwood and Verney (1954-55). In very careful clearance studies on the effect of oral water and saline administration in dogs they demonstrated that increases in the renal plasma flow and to a minor degree, in the glomerular filtration rate following water loading (2.5-3.0 per cent of body weight) exceeded the increases observed after loadings with 0.875 % of sodium chloride (2.4-3.0 per cent of body weight). These changes were independent of changes in the arterial pressure, actually this pressure was not seen to change during experiments in which the animals had been subjected to complete, or abdominal sympathectomy. Quite logically the authors argued that, if the increased plasma flow were the result of a reduced viscosity of the blood or an expansion of the glomerular capillaries (or as they might have included of the efferent vessels) accompanying the increase in blood volume it might have been expected - in contrast to their observations - that these parameters were changed more efficiently by saline loading than by water loadings. Based on this evidence they inferred that changes in the renal plasma flow and filtration rate are of intrinsic renal origin. Besides these increases preceded the increase in urine flow, occasionally decreasing again before the rate of urine flow had reached its peak, suggesting that the increases are associated with the induced changes in the water load. On the assumption that the increase in water content of the blood is not the primary (direct) cause of a fall in resistance of the arteriolar vessels we are driven to conclude that the slight increase in filtration pressure and therewith in filtration flow is the factor which is primarily responsible for the change in vascular resistance expressed in the observed increase in plasma flow. We infer therefore, that an initial increase in pressure within the tubule - acting in some unknown way and at an undetermined site - causes relaxation of the afferent arteriole and therewith a rise in glomerular capillary pressure. The initially effective increase in intratubular pressure must on this hypothesis be extremely small. The authors actually surmised that the macula densa region hypothetically might be the site in the tubule which through changes in the luminal pressure is controlling the afferent vascular tone; this theory was abandoned again because antidiuretic hormone which assumedly reduces the distal intratubular pressure if administered to animals in water diuresis has no appreciable effect on the renal plasma flow or filtration rate. It seems rather impressive how close to the present problem these authors arrived basing their deductions merely on data obtained in their clearance study. The suggestion that a vaso-dilator substance might be acting on the pre glomerular vessel is evidently based upon the (falsified) concept that the filtration rate is determined exclusively by the glomerular propulsive pressure. According to the present

concept the increase in the filtration rate and plasma flow observed as an event secondary to the increased intratubular pressure or some function thereof might equally well be caused by a decreased release of angiotensin. As observed, renal blood flow, the proximal Tm for salt and water and consequently the filtration rate will then increase.

Further evidence of the stimulus to the renin-angiotensin system has recently been reported by Vander and Miller (1964), whose results seem to be an important contribution to our present knowledge on this topic, their in-direct renin test may be subject to some objections though. In this respect the greatest importance should be attached to the fact that the incubated partly purified plasma samples and substrates used here apparently were not completely free from angiotensinase since a loss of about 25 per cent of added synthetic angiotensin was observed during incubation. Further, although probably of minor significance, the amounts of angiotensinogen in the incubation media were not checked. However, each one of these errors, if any, are prone to reduce the differences observed in plasma renin concentration of the renal venous blood in control and experimental periods. In the interpretation of their data it is of great importance that all results were obtained in acute experiments, thus minimizing the influence of late secondary adaptive mechanisms.

The renal venous plasma pressor activity, detectable in 85 per cent of normal dogs, was correlated to renal plasma flow (determined by the Fick principle using para-amino hippurate (PAH), creatinine clearance, urine flow, sodium plasma concentration – and excretion – and systematic arterial pressure). In control animals the renin concentration and arterial pressure, renal plasma flow, plasma sodium concentration and filtration fraction were not seen to be correlated. Some inverse relationship was suggested, however, between the renin level and creatinine clearance, although this correlation was statistically insignificant (corr. coeff. = 0.24,  $P = 0.2-0.1$ ), but a significant inverse relationship was found between the renin activity and the sodium excretion rate (corr. coeff. = 0.38,  $P \leq 0.04$ ). Taking into account the rather narrow range of variation in the creatinine clearance ( $1.67 \pm 0.43$  ml/min/kg BW) relative to that of sodium excretion rate ( $1.5 \pm 1.6$   $\mu$ M/min/kg BW), the relatively great variability involved in the clearance determination as opposed to the exactness and reproducibility of determinations of sodium excretion rate, as well as the rather insensitive bioassay method used, it can hardly surprise that correlation was not found significant between the renin plasma concentration and turnover rate of salt and water.

Renal venous plasma pressor activity increased significantly in 10 min

after partial constriction of the aorta above the renal arteries by which the mean femoral arterial pressure was reduced to 90 mm Hg. The activity was maximal 20–30 min after clamping and decreased again 10 min after re-opening of the arterial clamp. If however, diuresis was induced during aortic clamping, either as an osmotic diuresis (mannitol, urea or sodium sulphate) or by administration of chlorothiazide or acetazolamide, the increased pressor activity decreased again to pre-clamping values but neither plasma flow nor the creatinine clearance did return (increase) to control levels. If on the other hand the aorta was constricted during diuresis the increase in the pressor activity in the renal venous plasma always observed after clamping in non-diuretic animals, was eliminated. The diuretics however did not prevent the reductions in plasma flow or turnover rate of salt. Finally significant increases in plasma renin levels were found to follow high ureteral pressure (at least 60 mm Hg), induced by injection of urine into the renal pelvis via an ureteral catheter.

These results strongly support the view that a decreased pre-glomerular arteriolar pressure or distension per se is not the major stimulus to the renin-angiotensin system. The possibility that the pre-glomerular resistance in clamped animals might have been reduced by the diuretics, the effect being a compensatory increase of the distension (causing a reduced renin secretion) seems less likely since the renal plasma flow in these animals did not increase during diuresis. Vander's conclusion that the renin secretion is stimulated by a pressure reduction affecting some other functional parameter therefore seems justified. He further argues that the intratubular pressure or distension is not likely either to be the stimulus since renin output had been decreased by osmotic and other diuretics known to elevate this pressure but was increased after application of high ureteral pressure which would have a similar effect on tubular pressures. Thus according to Vander the most probable although hypothetical conclusions to be drawn from his results seems to be that the renin-angiotensin system is controlled by the flow or the composition of the distal intratubular fluid probably at the site of the macula densa. Although his results admittedly are suggestive of this conclusion it should be emphasized that the applied intratubular pressure during ureteral occlusion (from the study by Gottschalk and Mylle (1956) known to be parallel with renal pelvic pressures at levels above the normal intraluminal pressure of the distal tubules) was extremely unphysiological the intrarenal vascular resistances and distribution of pressure declines may also have been significantly disturbed under this condition whereby one experimental condition may become related to another but possibly uncomparable situation. Hence it

cannot be excluded completely that a normal response of the juxtaglomerular apparatus may have become reversed under this extreme condition

*To summarize* the many direct and indirect observations here presented concerning the conditions of a changed renin and/or angiotensin release it may be stated in conclusion that we remain ignorant of the adequate stimulus to the juxtaglomerular apparatus, but apparently changes in the afferent arteriolar pressure or distension can on no account be considered the major stimulus which rather may be some function of a change (reduction) in the glomerular propulsive pressure, whether this be the (reduced) flow the composition of the tubular fluid or – not very likely – the intratubular pressure or distension at the site of the macula densa

Vander and Miller (1964) did not suggest whether or how the resulting intrarenal actions of the liberated angiotensin might have a regulating feed back effect on the releasing stimulus. In this respect an additional comment should be made to their observations. It might be adequate to ask whether the present concept viz. that normal variations in filtration rate are determined by the tubular inhibitory action of angiotensin (increased release causing reduction in the filtration rate) has been invalidated by their observation that the glomerular filtration rate did not increase after the fall in plasma renin concentration after diuresis induced in partially clamped kidneys. This objection does not seem reasonable however since it is most likely that the proximal tubular reabsorption rate at the same time may have been inhibited by the osmotic action (creating a rate limiting concentration gradient) in case of osmotic diuresis and by a possible direct or indirect inhibitory tubular action of chlorothiazide and acetazolamide when these drugs were given.

A few of the more recent hypotheses on the function of the juxtaglomerular apparatus deserve a critical discussion. Thus Swann (1961) advanced the theory that the kidney balloon is not properly inflated at reductions of the perfusion pressure (e.g. by partial clamping of the renal artery). In compensation the kidney might liberate angiotensin which by its effect on the systemic pressure again might raise the distending pressure. It was suggested that there might be a pressor receptor as well as an effector endocrine gland in this circumscribed region containing a segment of the distal tubule and the end of the afferent arteriole. According to Swann's hypothesis the distension of the interstitial space is responsible for the control of the release of angiotensin. This concept is clearly incompatible with the observed fact that the proximal intratubular and peritubular capillary pressures are completely normal in most nephrons a few minutes after application even of



a rather tight partial clamp to the renal artery of rats (Leyssac 1964 b) In these kidneys the glomerular capillary pressures must have been significantly reduced (cf p p 85 and 89) further indicating that the intratubular pressure rather than the interstitial pressure is subject to regulation

Thurau (1964 b) advocates that the renin angiotensin system is involved in the renal autoregulation of blood flow and filtration supported by the observation by Waugh and Shanks (1960) who in isolated perfused kidneys found that the autoregulation was abolished when kidneys were perfused with colloid free solutions but recovered their autoregulation when plasma was added to the perfusion medium Thurau believes that the release of angiotensin is reduced as a consequence of reductions in the distal fluid sodium concentration at the site of macula densa This idea was based upon indications of low sodium concentration at this site, such as under conditions of osmotic diuresis in which vasodilatation occurs and on the finding by other investigators of an increased renin content in kidneys under conditions of sodium depletion reduced perfusion pressure to the kidneys obtained by clamping or by administration of pressor substances, and other conditions in which the sodium concentration at the macula site also must be reduced Consequently Thurau has to postulate first that liberation of angiotensin also is reduced under these conditions of increased renin content in the kidney, which is contrary to the direct evidence obtained (referred to above) Next he proposed that increased renal plasma flow at abrupt increases in renal perfusion pressure within the interval of autoregulation (80-100 mm Hg) initially would lead to increased distal tubular sodium concentration and flow

According to this hypothesis the increased sodium concentration would increase the liberation of angiotensin which again would reduce the plasma flow and filtration rate by an afferent vasoconstriction exclusively The author is of the opinion that the autoregulation and the glomerulo tubular balance is explained by this mechanism which opinion would be in accordance with the traditional interpretation of a proximal sodium reabsorption rate being load dependent Proximal sodium (- and fluid) reabsorption however must be described as a  $T_m$  limited process second Thurau postulates in this hypothesis that endogenously released angiotensin were to act on the pre glomerular vessel an idea which does seem most unlikely At the site of the juxta glomerular apparatus the afferent arteriole has lost more or less its muscular media the plain muscle cells being replaced by the epithelioid cells (the JG cells) in which myofibrils always are absent or extremely scarce Any continuous circular muscle layer which may act as a sphincter is not seen at this site A continuous muscle ring is not found until at the site of the efferent

arteriole (Faarup personal communication) Thus the first site for a vascular action of angiotensin released in the kidney must be distal to the pre glomerular vessel (An action on the glomerular capillaries is also unlikely because of the absence here of plain muscle cells and, angiotensin has been found to be a typical constrictor of plain muscle cells (Folkow, Johansson and Mellander (1961)))

Guyton Langston and Navar (1964) have also concerned themselves with the problem of renal autoregulation of the blood flow Several possibilities of feed back mechanisms at the juxtaglomerular apparatus have been taken into consideration and they have tested how predictions of these types of feed back according to the conventional concept of renal function fitted the data available in the literature finally they advocated the possibility of an osmotic feed back by which to control the afferent arteriolar resistance as the most likely type Based upon these considerations they presented a mathematical model of such osmotic feed back mechanism This model is based on the assumption that the post glomerular resistance is constant and the glomerular filtration rate proportional to the glomerular propulsive pressure and they postulated that the renal plasma flow varies directly with osmolality (at the site of macula densa) and with the arterial pressure Based on this model which thus involves untenable and unlikely assumptions they showed how computer predictions of the effect of varying different parameters of the model agree with known characteristics of renal function which finding they take as an indication that an osmotic feed back in the control of pre glomerular resistance could exist and be of significance to the autoregulatory phenomenon But they admit other types of feed back mechanisms should continuously be taken into consideration They believe that changes in the tubular fluid osmolality would change the contractility of the juxtaglomerular cells (by depolarization of the cell membrane) As discussed above these epithelioid cells may hardly be considered as contractile The idea that the renin angiotensin system might be involved in this autoregulating feed back mechanism was rejected because of the apparent conflict between the indications of vasodilatation during low arterial pressure to the kidney and vasoconstriction during conditions of sodium depletion both of which conditions are known to increase the release of angiotensin But to reject this idea merely on the basis of these arguments is hardly justified nor would it seem reasonable to consider mechanisms by which the intrarenal vascular resistances (and thus the blood flow) is regulated as a simplified system like the one proposed by Guyton here only one factor which acts solely on the pre glomerular arteriole is considered of significance Rather normally the regulation may be assumed to be a highly complex mechanism

and among other factors the afferent arteriole may be partly subject to nervous control in addition to its possible intrinsic myogenic, autoregulatory property, moreover the post glomerular tone may be controlled in part at least by an endogenous release of angiotensin. The relative importance of these many factors to the actual renal blood flow and resistances in any particular experimental or physiological situation can hardly be predicted at present.

It may be stated that the previous hypotheses on the function of the juxta glomerular apparatus are either unacceptable and/or insufficient as a means by which to correlate the likely stimulus to the effect elicited on the functional parameters of the kidney. A general objection to all of these hypotheses besides the criticism already mentioned is that they are incompatible with or do not account for the significance of a proximal Tm for salt and water and the tubular effect of angiotensin by which changes in the glomerular filtration rate normally in all probability are mediated and by which the proximal intratubular pressure and the fluid flow to the distal segments must be significantly influenced.

According to the hypothesis proposed by Leyssac (1964b) any reduction in the proximal intratubular pressure whether caused by a reduction in filtration pressure (i.e. glomerular propulsive pressure) or by a primary increase in the rate of reabsorption in this segment will increase the release of angiotensin by some still unknown change at the site of macula densa resulting from the minor decrease in the proximal hydrostatic pressure (the release thus being related to the distal load of salt). The rate of proximal reabsorption will thereby be depressed due to the tubular action of angiotensin reducing the glomerular filtration rate, and the efferent arteriolar resistance will be increased due to its vascular action. Both of these effects will support each other in restoring the proximal intratubular pressure towards normal levels and thus will counteract the angiotensin releasing stimulus.

This hypothesis of a feed back mechanism operating across the juxta glomerular apparatus seems to be compatible with the existence of a proximal Tm limited fluid reabsorption process and with the two primary actions of angiotensin. It may explain the regulation of the proximal intratubular pressure by which the fluid load to the distal tubular segments is maintained fairly stable and it is in accordance with the present evidence of a releasing stimulus to the renin angiotensin system. It is a serious question however whether the results obtained in experiments on the renal autoregulation are compatible with this hypothetical mechanism which presumably operates under physiological conditions. Although it is fully realized that this model can hardly be expected to explain all of the available data concerning the

subjects discussed in this review it still might be relevant to have the question discussed in this context. If a feedback mechanism of the above suggested type under normal conditions operates across the juxtaglomerular apparatus it might actually be predicted to react in some way or other under experimental conditions by which the perfusion pressure to the kidney is changed. Moreover indirect evidence of the participation of the renin-angiotensin system in the phenomenon of autoregulation was gained when a plasma factor (possibly angiotensinogen) was found to be of importance to the autoregulation (Vaughan and Shanks 1960). Further it has been demonstrated (e.g. Shipley and Study 1950, Thurau and Deetjen 1962) in semi-isolated kidneys *in situ* (cf p. 58) that the urine flow and salt excretion rate (osmolar clearance) increase almost exponentially when the renal perfusion pressure is elevated within the range 80 to 180 mm Hg in which range of pressure the glomerular filtration rates and renal plasma flows are maintained fairly constant. In these experiments the increase in water excretion at the high pressure levels amounted to about 8–10 per cent of the filtered volume. Taking into account the slowly variable and limited distal sodium  $\text{Tr}$  these data suggest (or at least do not exclude) a progressively diminishing proximal reabsorption rate during elevation of the perfusion pressure. Consequently, were the renin-angiotensin system to be involved in autoregulation these data might suggest that the release of angiotensin would increase with increasing arterial perfusion pressure (and glomerular propulsive pressure) apparently in contrast to the present hypothesis and the evidence of an increased release under conditions of reduced renal arterial pressure and negative salt balance. It should be emphasized however that such conditions by which the perfusion pressure to the isolated (denervated) kidneys is elevated by means of a pumping device is a rather extreme and unphysiological situation which hardly is comparable with normal conditions. The two different experimental situations may be complementary, each revealing one of two properties of the receptor system which cannot be observed together by the same procedure. Considered from this point of view the attention should again be directed to the anatomical arrangement of the juxtaglomerular apparatus especially to one of the characteristics of the three-dimensional models constructed by Barajas and Latta (1963) and Faarup (1964) (cf p. 76) demonstrating the fact that the ascending limb of Henle first approaches the post-glomerular arteriole of its affiliated glomerulus following it to the vascular pole of the glomerulus where it contacts the afferent vessel running along with this pre-glomerular vessel throughout a certain length. This characteristic counter-current system between the distal tubule and the arterioles common for all nephrons is highly suggestive of the fact

that the arteriolar system in some way may be of significance also for the 'receptor' mechanism at the juxtaglomerular apparatus possibly as a reference system against which stimulating changes in flow fluid composition or pressure in the distal lumen are perceived. If this speculative concept may be accepted as a working hypothesis an increased output of angiotensin under conditions of increased perfusion pressure to the isolated (denervated) kidney may not be implausible nor contradictory. Under such conditions the reference would be seriously changed possible to such a degree as to cause an apparently inverted response to an otherwise normal stimulus by which response the normal regulation of the intratubular pressure effectuated by angiotensin consequently would break down. Actually the renal tissue pressure (representing the proximal intratubular pressure) is completely uncontrolled under such experimental condition and changes in parallel with the arterial perfusion pressure as shown by Hinshaw and his group (1964) (cf p 25). This situation is probably in direct opposition to more physiological conditions of elevated arterial pressure, imitated by induction of neurogenic hypertension, in which the elevated arterial pressure, in part at least, is caused by increased arteriolar resistance. In this latter case the pressure or flow at the end of the afferent arteriole would probably be unchanged or even moderately reduced. All is a normal response from the juxtaglomerular apparatus and a normal pressure regulating effect of angiotensin should be anticipated. Accordingly Thureau and Wober (1962) found the proximal as well as the distal intratubular pressures to remain unchanged in rats which seem to have been under this experimental condition. Thus the data obtained concerning autoregulation may seem to be fully compatible with this modification of the hypothesis suggested by Leyssac (1964b) according to this modification the arteriolar system will act as a system for reference towards a normal angiotensin releasing signal acting from the luminal side of the distal tubule at the site of macula densa. In this modification of the model a more complete functional correlation with the structural arrangement seems also to be obtained.

Finally it should be mentioned that even though the antidiuretic hormone reduces the distal intratubular pressure when administered to water diuretic animals as shown first by Wirz (1956) which effect is known to be without influence on the proximal turnover rate of salt and renal plasma flow this fact does not necessarily invalidate the present interpretation and model (cf Seliwood and Verney 1954-55 referred to p 100) the possibility that the distal luminal pressure or distension may represent the adequate stimulus to the renin-angiotensin system need not be invalidated although it provides a further evidence against such possibility. The possibility is still open that these

two octapeptides (angiotensin and vasopressin) may have an antagonistic (competitive?) action on the proximal reabsorption process in mammals. In fact some – although not conclusive – evidence of the enhancing effect of vasopressin on the proximal sodium transport in mammals has been presented by Clapp, Watson and Berliner (1963). By micropunctures they showed that about 57 per cent of the filtrate had been reabsorbed at the midpoint of the proximal tubules in antidiuretic dogs as evaluated from  $\gamma$  F/P inulin of 2.3. In water diuretic animals (3–4 hours after initiation of the diuresis) only 38 per cent (F/P inulin 1.6) of the filtered volume was reabsorbed at this site of the tubule. In both conditions the proximal tubular fluid was isosmotic with dog plasma. In similarly hydrated dogs infused with vasopressin (100 mU per kg of body weight per hour) the mean F/P inulin was 2.0 in the middle third of the proximal tubules (equal to a fractional reabsorption of 50 per cent). These ratios differed significantly. Although the mean inulin clearances were claimed not to differ in the diuretic and non diuretic groups, possible changes in the absolute rate of reabsorption could however not be evaluated in these experiments as emphasized also by the authors because of the great variability in the clearance in dogs – a rather small number of animals investigated and the absence of individual control measurements before hydration nor was a possible vascular (preglomerular) action of the infused vasopressin excluded in these experiments in which measurements of plasma oncotic pressures and of possible changes in the proximal intratubular hydrostatic pressures also might have been of interest. Along this line of argumentation attention should be directed to the fact that clearances of inulin were about 50 per cent lower than normal in rats with diabetes insipidus (Gertz, Kennedy and Ullrich 1964). In these (unloaded) rats the fractional reabsorption did not differ significantly from controls thus the proximal reabsorption rate had been depressed suggesting a relatively increased sensibility to endogenous angiotensin. The question cannot be answered, however, until the adequate stimulus to angiotensin and the possible influence of vasopressin action on this stimulus or on the effector sites has been clarified in detail. These problems will therefore be important subjects for future research.

*In summary*, data forming the basis of the present review may be interpreted by the following hypothetical sequence of events. A condition of decreasing pre glomerular pressure (e.g. provoked by gradual obstruction of the renal artery) and an initial state of high intrarenal turnover rate of filtrate may be considered as an illustrating example. By partial obstruction of the artery, thereby lowering the glomerular propulsive pressure, the proximal intratubular pressure will decrease initially. Hence some function of this decreased pressure at the site of the macula densa, possibly amplified during

flow through the loop of Henle, will stimulate the release of angiotensin. Consequently two effects will follow. The proximal reabsorptive capacity for salt and water will be depressed and the post glomerular arteriole be constricted. Both of these primary, intrarenal effects will tend to restore to normal the proximal intratubular pressure and the glomerular filtration rate will decrease. Also the renal blood flow will be diminished because of the reduction in the pre glomerular pressure and the increase in the post glomerular resistance. With a further reduction in the propulsive pressure the release of angiotensin will increase until a maximum effect on the proximal  $T_m$  is reached. Even if more angiotensin were released with further obstruction of the renal artery, the reabsorption rate would not be further depressed. Consequently an increased fraction of the filtered volume is reabsorbed proximally and the load to the distal tubular segment will be diminished. Hence the distal intratubular pressure will fall, as also demonstrated in such partially clamped kidneys (cf p 89), and the urine flow decrease (cf p 85). The reduced internal diameter of the distal tubule (equal to increased resistance to fluid flow) also observed under these conditions (Leyssac 1964 b) explains readily how the proximal intratubular pressure still can be maintained in spite of a decreased luminal flow rate. If a further drop in the glomerular propulsive pressure occurs less fluid will finally be filtered than the proximal tubule is able to reabsorb and the luminal flow velocity will reach zero value upon which the proximal lumen occludes.

It should be emphasized again that the most essential resultant effect of this regulating mechanism is the fairly constant supply of salt to the distal part of the nephron maintained irrespective of such variations in the glomerular filtration rate as are provoked by stimuli to the highly reactive pre glomerular vascular system or by primary metabolic changes in the tubular transport capacity, both of which may be unrelated to the salt balance.

In view of the present concept a number of other experimental data may be predicted or comprehended some of which shall be briefly discussed in the following chapter.

## CHAPTER VI

### Some further observations relevant to the present hypothesis

#### a) *Hypothetical considerations on the location of the tubular action of angiotensin*

It has appeared from the previous chapters that the tubular action of angiotensin is a depression of the rate by which the proximal fluid volume is reabsorbed (a depression of a volume  $T_m$ ). Consequently its action need not specifically be an inhibition of the sodium transfer rate ( $T_m^{Na}$ ) but rather an inhibition of the  $T_m$  of all the predominant electrolytes and urea (cf p. 67). Such action on the volume transfer rate without an appreciably changed composition of the proximal luminal fluid conforms closely with the concept that the tubular action of angiotensin serves to stabilize the proximal hydrostatic intratubular pressure in order to maintain a fairly stable supply of salt to the distal tubular segment. Further according to the hypothesis previously proposed (Ch. II) an inhibition of the rate of proximal fluid reabsorption would be equal to an inhibition of the normally rate limiting luminal micropinocytosis. It might therefore be tempting to suggest that the tubular site of action of angiotensin were localized to the luminal brush border membrane. This suggestion is not inconsistent with the concept deduced from studies on pinocytosis in amoebae (e.g. Chapman and Andresen 1962; Chapman, Andresen and Holter 1964) according to which pinocytosis (which may be induced only by charged molecules as e.g. sodium chloride, amino acids and proteins in solution) is induced by an initial adsorption of the inducer to the external mucopolysaccharide layer of the plasmalemma, which as a secondary event initiates channel formation. The mammalian brush border is known to contain mucopolysaccharides as indicated by its staining reaction with periodic acid Schiff (PAS positive), and further that angiotensin is reversibly bound by heparin (a polysaccharide sulphuric ester) as shown by Jacques et al. (1960). Thus it is not unlikely that angiotensin has a rather specific affinity to the luminal membrane. Further it should be mentioned that angiotensin



even in very high concentrations has no influence at all on the oxygen consumption of incubated rat kidney cortical slices (Leyssac, Lassen and Thaysen unpublished), nor has it any effect on the enzyme activity of a Na K activated adenosin triphosphatase system isolated from mammalian kidney cortex (R. L. Post 1961 personal communication Bonting Canada and Hawkins 1964). Although these latter observations hardly lend any support to the suggestion they are not incompatible with the theory that the angiotensin action is localized to the luminal step in the transfer process

#### b) *Glomerular filtration rate and distal salt load*

One of the consequences of a regulation of the salt load or flow velocity to the distal tubular segments would be the well established lack of correlation between the intrarenal turnover rate of salt and the electrolyte excretion rates which is rather difficult to understand on the basis of the previous concept of a load dependent proximal reabsorption rate determined by the rate of filtration. According to this (latter) concept any increase in the filtration rate should result in a more abundant supply of sodium to the distal parts of the nephron (on the assumption of a constant fractional reabsorption proximally). If the reabsorptive capacity of the distal nephron segments is limited which seems to be a well established fact the rate of sodium excretion would vary as a certain nonlinear function of the filtration rate (cf Pitts 1963, p 191). Were this always the actual case the following observations are difficult to explain ■ ■ ■ that administrations of growth hormone, repeatedly found to increase considerably the inulin clearance, induce sodium and potassium retention even in adrenalectomized animals (Stein et al 1952). According to the concept advocated here, hormonal or other metabolic stimulators primarily increasing tubular reabsorptive capacity enhance the glomerular filtration rate by their tubular effect lowering the proximal intratubular (capsular) pressure. This effect which will reduce the distal load of sodium is counteracted by an increased release of angiotensin by which the tubular fluid flow is largely maintained although not completely. In conformity with this Thurau (1964 c) and associates showed that infusion of metabolites of adenosine triphosphate (ATP) as adenosine and adenylic acid which increase the metabolic activity and exert a vasodilating effect in other organs elicit an instantaneous reduction in the renal blood flow in dogs. Thus according to the present hypothesis the direction of the changes in the distal load which accompany increases in the glomerular filtration rate will depend on whether the primary cause of this increase is of vascular or tubular origin.

c) *The 'dilution diuresis' and expansion of the extracellular fluid volume*

From a similar point of view the sequence of secondary events after rapid infusions of Ringer solutions as described by Bojesen (1954b) (cf Ch III B a, p 59-60), may be interpreted in greater detail. The immediate increase in the salt and water excretion rate following reductions in the oncotic pressure of the plasma (phase I), previously discussed in detail is explained most readily by the accompanying increase in the intratubular pressure before the proximal reabsorptive capacity has been changed. The second phase - a measurably increased renal plasma flow, proximal reabsorption rate and rate of filtration -, was always seen 10-15 min after such expansions of the blood volume whether or not changes in the oncotic pressure were provoked, probably this is a consequence of a depressed release of angiotensin induced somehow by the effect of the elevated proximal tubular pressure which will follow reductions in the oncotic pressure and/or reductions in pre glomerular arteriolar resistance likely to occur as a response (neurogenic) from extrarenal volume receptors. Moreover, the changed angiotensin output from the kidney influences assumedly the adrenal cortical secretion of mineralocorticoid hormones since it has been demonstrated repeatedly in recent years that angiotensin plays a dominant role in the physiological regulation of aldosterone secretion. As a detailed discussion of the many data reported in the literature concerning this complex problem is beyond the scope of this review only a few of the established facts shall be mentioned.

It has been demonstrated unequivocally that infusion of angiotensin increases the aldosterone excretion and secretion rate in man (Genest et al 1960, Laragh et al 1960a) in the dog (Mulrow and Ganong 1961), and in sheep (Blair West et al 1962) also when injected directly into the adrenal artery this fact is now used as a test for the specificity of the angiotensin yield in the indirect renin assay. In vitro stimulation by angiotensin of aldosterone production from adrenal cortical slices was shown by Kaplan and Bartter (1962) and Davis and his group has shown that the development of secondary aldosteronism following thoracic cava inferior constriction in hypophysectomized dogs was abolished by nephrectomy while removal of a series of other organs had no such effect (Davis et al 1961). Subsequent studies from these and other groups have confirmed the close interrelationship of angiotensin - and aldosterone plasma levels and salt balance (cf Ch V, B), several investigators consider angiotensin as the trophic hormone to aldosterone secretion (e.g. Laragh, Cannon and Ames 1964, Davis 1964). But the problem is complicated by the demonstration first by Laragh and associates (1960) that the aldosterone secretion rate in spite of sodium depletion

uninfluenced in patients with depressed plasma potassium concentration. On correction of the potassium loss by oral administration, the plasma potassium concentration rose abruptly as did simultaneously the aldosterone secretion rate. Further evidence of the importance of potassium to aldosterone secretion was provided by Blair West and his group (Blair West et al 1962) who in their sheep preparation in which an adrenal gland was transplanted to the neck in order to infuse directly into the adrenal arterial supply and to collect adrenal venous blood showed that sodium loaded animals in contrast to un-loaded animals did not respond to infusion of small doses of angiotensin with increases in the aldosterone and corticosterone secretion. However, infusion of solutions containing a high potassium to sodium concentration ratio did elevate the aldosterone and corticosterone secretion in these loaded sheep without any appreciable effect on the cortisol secretion rate. At the C I O M S meeting in Prague (aug 1963) R. E. Peterson reported also that administration of angiotensin to rats had failed to provoke an aldosterone stimulation which fact is not explained by a high blind value of plasma aldosterone (great stimulation of endogenous angiotensin) due to the operative manipulations since similar results were obtained in salt loaded rats in which the blind aldosterone plasma concentration was close to zero (Bojesen, personal communication). These observations emphasize that other (inhibitory or competitive) factors may become of great significance to an adequate response of the adrenal cortex to stimulation by angiotensin. This fact would seem reasonable and might actually be anticipated, since an invariable increase in the aldosterone secretion rate caused by angiotensin even when released because of influences unrelated with the salt balance would result in salt retention whether the animals were in a state of negative salt balance or, as in the above experiments even were salt loaded.

It still seems safe to state that angiotensin plays a major role as a physiological stimulator of the aldosterone secretion. At present this statement seems to be the most reasonable clue to an interpretation of the third phase in dilution diuresis secondary to expansions of the extracellular fluid space, viz a prolonged and moderate increase in the rate of salt excretion with increased sodium to potassium concentration ratio in the urine observed some 45-60 min after such expansions (Bojesen 1954b). This observation is to be expected from the depressed release of angiotensin inferred from phase II by which the rate of aldosterone secretion would also be depressed. Further evidence in favour of this interpretation was provided by Bojesen and Degn (1961). These authors demonstrated that plasma levels of aldosterone had increased markedly within 10 min in normal dogs after blood volume depletion (bleeding) and again decreased markedly at a similar rapid rate after

volume expansion (transfusion) This observation might suggest indirectly that the third direct effect of angiotensin (the adrenal effect) is as rapid as its two intrarenal effects

d) *The renal effect of noradrenaline and other pressor substances*

In clearance studies on the effect of noradrenaline and of angiotensin on salt excretion it has repeatedly been shown that the renal response to these pressor substances is qualitatively similar The typical acute effect in human subjects and dogs is a reduction in urine flow sodium excretion rate and renal plasma flow with a more moderate reduction in the inulin clearance These effects have generally been ascribed solely to the vascular action of the substances which interpretation cannot be maintained since the Tm limited character of the proximal fluid reabsorption and the tubular effect of angiotensin on this Tm has been demonstrated directly Changes in the proximal reabsorption rate accompanying changes in renal plasma flow after infusions of noradrenaline therefore must be ascribed either to a rapid direct tubular action of noradrenaline or to a secondary effect mediated by a rapid intrarenal regulatory mechanism Since such direct and instantaneous effect of noradrenaline on the occlusion time in rats could not be demonstrated (Leyssac 1965 b) the changed renal tubular reabsorption rate was inferred to be a secondary event following the administration of noradrenaline In consistency with this inference Scornic and Paladina (1964) (cf p 95) observed significant increases in the plasma concentration of angiotensin following infusions into dogs of 2-4  $\mu\text{g}$  of noradrenaline per kg of body weight per min Further it is a well established fact that a paradoxical response may be observed also in acute clearance experiments in dogs and human subjects receiving small amounts of noradrenaline (vide infra) As discussed by Kruhoffer (1960) a rise in systemic arterial pressure resulting from the profound effects of the substance also on other parts of the vascular system may counteract the decline in glomerular propulsive pressure produced by a pre dominant pre glomerular vasoconstriction The significance of this possibility has clearly been demonstrated by Langston et al (1962) They showed in areflex dogs (complete spinal anaesthesia) that the renal blood flow increased as a result of the increase in arterial pressure at infusion rates below 0.6  $\mu\text{g}$  of noradrenaline per kg of body weight per min Above this infusion rate the renal effect of noradrenaline caused the blood flow to decrease notwithstanding the continuous rise in arterial pressure The renal blood flow remained uninfluenced by infusions at the low rates if a constant renal arterial pressure was maintained by means of a controlled arterial constriction moreover when the

renal arterial pressure was not maintained constant but even increased renal blood flow would decrease at the high dosage levels. Changes observed in creatine clearance and urine flow rate were seen to be parallel to those seen in the blood flow. If noradrenaline was infused at a low rate the rise in glomerular filtration rate (10–15 ml/min) would be seen to exceed by far the rise observed in urine flow (10–15 ml/min). Thus, the rate of proximal reabsorption obviously increased during the infusion of small amounts of noradrenaline. A rise in renal blood flow and propulsive pressure, as a result of infusion of small amounts of noradrenaline per unit of time would tend to increase the proximal intratubular pressure and thereby salt and water excretion rates. According to the present hypothesis the result will be that less angiotensin will be released because of this increase in pressure thereby increasing the rate of reabsorption proximally, and vice versa during infusions of noradrenaline at rates above 0.6  $\mu$ g per kg of BW per min. Thus the finding reported by Langston and collaborators seems to fit the present hypothesis. This hypothesis is also commensurate with the observation of a moderate reduction in the excretion rate of potassium and a simultaneous moderate increase in the excretion rate of sodium in response to prolonged but small rises in the blood concentration of noradrenaline provoked by intramuscular injections of the substance in oil (Duncan et al. 1951, Bliss, Rubin and Gilbert 1951). The significance of this finding which apparently represents the reverse of an aldosterone effect, has hitherto seemed obscure. However, according to the above reasoning a decreased release of angiotensin should be predicted as a consequence of such small increases in the blood level of noradrenaline. Since angiotensin seems to be of major importance to the regulation of aldosterone secretion (cf. section b, of this chapter), a reduction in the secretion of aldosterone should represent a secondary event in a prolonged administration of small doses of noradrenaline.

Similar arguments may be valid also in cases of other pressor substances the administration of any pressor substance in doses which will affect the glomerular propulsive pressure in some way or the other may be predicted to influence immediately the signal to the intrarenal control mechanism at the juxtaglomerular apparatus thus causing a change in the release of endogenous angiotensin.

e) *The importance of a voluminous proximal fluid reabsorption in mammalian kidneys*

A functional characteristic of mammalian kidneys which has puzzled many physiologists is the apparent peculiarity that such voluminous amount

of plasma has to be filtered and reabsorbed again predominantly in the proximal segment of the nephron by an energetically expensive transport process in order to secure the excretion of an amount of final urine corresponding only to 1-2 per cent or less of the filtered volume. However, having once realized that a prerequisite of a controlled excretion rate of salt seems to be a fairly constant load of salt to the distal segments determined by the hydrostatic pressure difference between the proximal and distal tubules (cf p 87-88) a rapid control mechanism by which a fairly stable proximal intratubular pressure can be maintained seems to be of evident significance especially in homothermic animals with a highly reactive vascular system. Since such regulating mechanism hardly will be sufficiently stable and accurate if it were to be effectuated solely by an adjustment of the efferent arteriolar tone, an adjustment of the proximal reabsorption rate seemed to be required in order to provide to this pressure an additional stabilizing effect (cf p 90-91) which effect most likely is provided by the tubular action of angiotensin. No doubt the efficiency of the regulating intrarenal mechanism will depend on the capacity of proximal fluid reabsorption and filtration. Since the glomerular capillary hydrostatic pressure in mammals may vary considerably (probably over a range of some 5-10 mm Hg), a small capacity and thus range of variation of the proximal reabsorption relative to such change in the glomerular propulsive pressure would greatly limit the efficiency of the regulation. On the other hand the great capacity and range of variation of the proximal reabsorption actually present in mammalian nephrons has opened the possibility of a highly effective pressure regulation and thereby of a stable and regulated salt excretion. It is apparently in keeping with this concept of a regulation of the load of salt to the distal nephron segments that changes in this load serve as the adequate stimulus to the control mechanism.

#### *f) The hypothesis considered in the light of evolution*

Finally it is in keeping also with the above mentioned concept that a juxtaglomerular apparatus containing the renin-angiotensin system apparently is found only in homothermic animals. Bean (1942) reported that renin found in kidneys of several mammals and birds could not be detected in kidneys of poikilotherms (fish, amphibia or reptiles) although these latter animals actually were seen to react with pressor response to renin extracted from mammalian kidneys. It might be interesting to have the result of extensive studies on this subject, using one of the more sensitive and specific renin assays developed in recent years, any definite conclusions can hardly be drawn until such results are available. If the suggestion inferred from

Bean's report is found to be tenable it might be suggested that a renin containing juxtaglomerular apparatus had evolved concurrently with the loop of Henle and a warm blooded state which would seem meaningful in the light of the present hypothesis. Water conservation and sodium conservation are equally essential in animals with a terrestrial habitat. The ability to make a urine which is more concentrated than the plasma which must have been part of the adaptation to terrestrial life is seen only in birds and, to a much greater extent in mammals only because bird and mammal tubules are bent into the loops of Henle. In birds most of the nephrons are of the reptilian type and only few have a loop. In mammals all nephrons have a loop, the relative length of which is closely correlated with the concentrating ability of the integrated kidney (B. Schmidt Nielsen and O. Dell 1961). In mammals, which excrete their nitrogenous waste products as the osmotically active urea in contrast to the almost insoluble uric acid excreted by birds and reptiles a more efficient concentrating mechanism would also seem to be required in order to reduce the loss of water accompanying urea excretion especially in mammals adapted to an arid habitat. Such desert mammals including desert rodents, the urine of which may achieve osmotic concentrations of 5.5-6.5 osm/l (cf. K. Schmidt Nielsen 1964 table XXVI) may also excrete an excess of water in a hypotonic urine when drinking water is available. Similarly an efficient sodium conserving mechanism permitting also an excretion of excess of sodium and operating independent of the simultaneous water excretion would appear to be equally essential for the survival of mammals. This requirement is further stressed in mammals in which regulation of the salt excretion is taken over entirely by the kidney (in contrast to the additional extrarenal salt excretion by salt glands seen in birds and reptiles), and which have no possibility of regulating the salt intake either by the oral or the percutaneous route. Such efficient regulation would seem to be conditioned by a controlled salt load to the distal tubular segment in which the final regulation of the excretion rate takes place.

In this context it should be mentioned also that a fairly stable tubular flow velocity in the loop of Henle is highly important for the concentrating ability of the counter current multiplier system in this structure. The counter current hypothesis implies that the maximum concentration to be established by the system will be reduced by marked increases as well as decreases in the flow rate. If flow rates are high because the concentration difference between the two limbs (which create the multiplication) will not be fully established on account of the insufficient time of contact with the fluid, in the case of very low flow rates because of back diffusion of water from neighbouring areas in which fluid is less concentrated. According to Kuhn

(1962) a reduction in the flow rate in the loop by a factor of 10 would seriously impede the multiplication of osmotic concentration because of back diffusion. However it should be emphasized again that it still remains an unanswered question to which extent the juxtaglomerular apparatus exerts a regulating influence on the proximal intratubular pressure in the juxta medullary nephrons which predominantly are engaged in the counter current system due to the long loops of Henle as opposed to the short loops and relatively long proximal convolutions of most nephrons in the outer cortex of many species. In some species (certain desert rodents, Sperber 1944) in which all nephrons have long loops of Henle and for this reason probably contribute equally much to the counter current system and the salt excretion the regulating effect of the renin angiotensin system on the intratubular pressure might probably be of importance also in the counter current system. On the basis of our present knowledge however it is too early to enter into a more detailed discussion and interpretation of the integrated renal function.

On the basis of the above reasoning it may be suggested tentatively that the juxtaglomerular apparatus is specifically evolved as a simple functional structural arrangement in the mammalian nephron concurrently with and interdependent with the loop of Henle thus providing a basis for a sodium conserving mechanism to operate in the distal segments as does the loop arrangement for a water conserving mechanism also to operate in these segments.



## Final remarks

The purpose has been to review a few data, considered to be essential to the comprehension of the problem at issue the accomplishment and regulation of the proximal tubular reabsorption in the mammalian kidney, which problem is considered highly important if we are to understand the regulation of renal salt excretion and the extracellular fluid space. The data presented have driven the author to dispute and reject some of the current assumptions and to propose instead alternative hypotheses with respect to the regulation of the reabsorption rate and the mechanism of transcellular transfer of the reabsorbate. The impression may have arisen that the bulk of data might be comprised in a unifying concept, thus bringing into accord data obtained from studies on the integrated function of the whole kidney, on the single nephron and the proximal cell and on the transport characteristics of the individual constituents of the reabsorbate. This functional concept seems largely to be in accordance with existing macroscopical and ultramicroscopical structural equivalents.

It is my hope that the theory presented here, which only should be regarded as a rough sketch, may stimulate future research and conceptual approaches for future design of experiments which eventually may disclose new and additional physiological aspects. Many of the details of and inferences from this hypothesis require a more exact formulation and further experimental verification, which no doubt will lead to modifications and further elaborations of the proposed concepts.

# Appendix Statistical analysis

by

*Inger Harder Hansen*

## *Statistical analysis*

The present study concerns the relationship between the clearance of inulin, denoted by  $(v)$  and the reciprocal of occlusion time  $(u)$

$(v)$  has been measured 2-4 times in each rat hence it is possible to derive an estimate of the variance of  $(v)$ . Contributors to this variance are changes in the rate of reabsorption during the collection periods and experimental errors. When the mean value of  $(v)$  and the value of  $(v)$  in the last collection period, respectively, are plotted against  $(u)$  in each experiment the points are seen to scatter most in the latter case. It therefore seems reasonable to use the means for a comparison with  $(u)$ . Since repeated measurements of the occlusion time are not available, the variance of  $(u)$  cannot be estimated.

In fig 1 (p 75) the mean of the measured values of  $(v)$ , denoted itself by  $(\bar{v})$  is plotted against  $(u)$  in the group of control rats. It seems reasonable to claim a linear relationship which may be derived by the method of linear regression analysis with  $(v)$  as independent variable, thus disregarding its variance. The assumptions necessary for the application of this method are that the observations are independent and that  $(u)$  is normally distributed for each value of  $(v)$  with the same variance. The constancy of the variance can roughly be checked by grouping the observations according to  $(v)$  and computing the variance within each group. Any tendency of the variance to decrease or to increase with  $(v)$  could not be demonstrated by this test and the values were reasonably constant. When the assumptions regarding normality and constancy of variance and the linearity were further checked they were found to be fulfilled in all cases.

In the further analysis data  $(v) > 1.60$  are disregarded since they are few only and scattered.

The common estimate of the relationship between  $(u)$  and  $(v)$  for the control rats is  $y = 5.82 + 4.582 (v - 1.091)$  with  $s^2 = 0.624$   $s = 0.0085$ , and  $s_b = 0.164$  each with 69 degrees of freedom. A  $t$  test shows that this line does not differ significantly from the line through  $(\bar{v}, \bar{u})$  and  $(0,0)$  i.e.  $y = 5.33$

(v) The relationship between (u) and (v) in control rats with (v)  $\leq 1.60$  may therefore be described by  $(u) = 5.33 (v)$  in which the estimate of the slope has the variance  $s_b = 0.0071$  and  $s = 0.670$ , each with 69 degrees of freedom. This line is given in all the figures.

### *Dose response of angiotensin*

The data concern the relationship between inulin clearance and occlusion time in rats given a dose of ( $d_1$ ) ng of synthetic angiotensin in the interval between the measurements of inulin clearance and occlusion time. It is obvious from the data that angiotensin prolongs the occlusion time. Hence the clearance of inulin is denoted by  $v(d)$  and the reciprocal of the occlusion time by  $u(d)$  ( $d$  indicating the total dose of angiotensin (exogenous plus an endogenous equivalent) corresponding to its concentration in the renal peritubular plasma at the moment concerned).

It was found above that the relationship between  $u(d)$  and  $v(d)$  might be described by  $u(d) = \lambda v(d)$ , with  $\lambda = 5.33$  as an estimate of  $\lambda$  having  $s^2 = 0.0071$ .

On the assumption that the physiological variation in (u) is caused by an equivalent to endogenous angiotensin ( $d_1$ )  $u(d_1 + d_2)$  is plotted in fig 2, 3 and 4 against  $v(d_1)$  for ( $d_2$ ) equal to 25 ng, 7.15 ng and 5 ng respectively.  $v(d_1)$  is represented by the mean of repeated measurements, assuming that ( $d_1$ ) remains reasonably constant throughout the observation period. The purpose is to describe a response curve  $y = v(d)$ . The function  $v(d)$  generates the curves around which the points in the fig 2-4 scatter, since it for any observed  $v(d_1)$  determines ( $d_1$ ) and for ( $d_1 + d_2$ ) determines  $v(d_1 + d_2)$ . Hence  $u(d_1 + d_2) = \lambda v(d_1 + d_2)$  giving a functional relationship between  $v(d_1)$  and  $u(d_1 + d_2)$ .

Fig 2 suggests a dose response relationship with horizontal asymptote  $y = \alpha$  giving

$$(1) \quad v(d) = \beta e^{-\gamma d} + \alpha$$

in which  $\alpha$ ,  $\beta$  and  $\gamma$  are unknown positive constants. From this equation we obtain the following expression for  $u(d_1 + d_2)$  as a function of  $v(d_1)$

$$(2) \quad u(d_1 + d_2) = \lambda v(d_1 + d_2) = \lambda (\beta e^{-\gamma(d_1 + d_2)} + \alpha) = \\ \alpha \lambda (1 - e^{-\gamma d_2}) + \lambda e^{-\gamma d_2} \times v(d_1)$$

An exponential dose response relationship of this form would yield straight lines with the slopes  $(\lambda e^{-\gamma d_2})$  passing through  $(\alpha, \alpha \lambda)$ . But the registered

data do not fit strictly with straight lines for all the doses investigated and the estimate of  $(\alpha) - (\alpha)_{\infty}$  derived from the regression lines fitted to the observed data, was somewhat greater (0.949) at a dose of 5 ng than that estimated at the two other dose levels (0.805 and 0.836 respectively). This inconsistency emphasizes the fact that the dose response curve is not exactly the one expressed in equation (1). However, since the difference in the estimate of  $(\alpha)$  is not very great this equation has been chosen as a first approximation to a quantitative description of the data. In order to find a usable approximation to a dose response curve of the form (1)  $v(d) = \beta e^{-\gamma d} + \alpha$ , we have to determine  $(\alpha)$  and  $(\gamma)$  from the three straight lines shown in fig 2-4 which agree reasonably well with the observed data. The best approximation seemed to be that  $(\alpha) = 0.83 \text{ ml/min/g h.w.}$  and  $(\gamma) = 0.2 \text{ ng}^{-1}$  using these values the data are described by the regression lines given in the Table I (p. 79) and shown in figs 2-4.

Although the estimates of  $(\alpha)$  and  $(\gamma)$  are rough only the curve given by

$$(1a) \quad v(d) = \beta e^{-0.2d} + 0.83$$

seems to give a reasonable approximation to the dose response curve.

The parameter  $(\beta)$  cannot be estimated on the basis of the present data since the relationship between  $u(d_1 + d_2)$  and  $v(d_1)$  does not include  $(\beta)$ .

The reduction in glomerular filtration rate caused by administrations of  $(d_2)$  ng of exogenous angiotensin to a rat (250-300 g of body weight) at a state of function corresponding to the equivalent of  $(d_1)$  ng may be calculated as a percentage of the maximally possible reduction from the above dose response relationship as follows

$$(3) \quad \frac{v(d_1) - v(d_1 + d_2)}{v(d_1) - \alpha} = \frac{\beta e^{-\gamma d_1} + \alpha - (\beta e^{-\gamma(d_1 + d_2)} + \alpha)}{(\beta e^{-\gamma d_1} + \alpha) - \alpha} = 1 - e^{-\gamma d_2}$$

Thus the exponential dose response function implies that the effect relative to the maximum response at any value of  $(v(d_1))$  is independent of  $(d_1)$ .

For  $d_2 = 5 \text{ ng}$  the effect is  $= 1 - e^{-0.2 \times 5} = 63.2 \%$

For  $d_2 = 7.15 \text{ ng}$  the effect is  $= 1 - e^{-0.2 \times 7.15} = 76.1 \%$

For  $d_2 = 25 \text{ ng}$  the effect is  $= 1 - e^{-0.2 \times 25} = 99.3 \%$

Solving the above equation (3) with respect to  $(d_2)$  we get

$$(4) \quad e^{-\gamma d_2} = \frac{v(d_1 + d_2) - \alpha}{v(d_1) - \alpha} \text{ and consequently}$$

$$(5) \quad d = -\frac{1}{\gamma} \log_e \frac{v(d_1 + d) - \alpha}{v(d_1) - \alpha} = -\frac{1}{0.2} \log_e \frac{v(d_1 + d) - 0.83}{v(d_1) - 0.83}$$

Equation (5) expresses the amount of exogenous angiotensin in ng required to reduce the clearance of inulin in a rat kidney from  $v(d_1)$  ml/min/g KW to  $v(d_1 + d)$  ml/min/g KW, using intravenous application

In Table II (p. 82)  $(d_2)$  is calculated for a few values of  $v(d_1)$  and  $v(d_1 + d_2)$

## Summary

The establishment of the general, biological requirement of salt balance in the organism, reflected in the maintenance of a stable volume of the extracellular fluid space depends in mammals almost exclusively on a regulation of the renal salt excretion

If the mechanisms involved in such regulation of the renal excretion were to be disclosed and comprehended, a prerequisite would be that methods were developed which permitted a clear distinction between vascular and tubular factors in the renal handling of salt and water. The review presented here is concerned mainly with data obtained from studies on the reabsorption of filtrate by the proximal tubules of mammalian nephrons in which tubular segment the great majority of filtered salt and water is reabsorbed. This topic was chosen for the experimental investigation, on which this review is based because it was not generally agreed whether the rate of reabsorption of salt and water in this tubular segment is dependent or independent of the filtered load of salt. The main theme of this review is the analysis of this problem and its implications which is of fundamental importance for the entire concept of the mechanisms involved in urine formation and especially for the understanding of the mechanisms involved in the regulation of the renal salt excretion.

*In the first chapter* some early contributions to the understanding of the fundamental features in urine formation are briefly mentioned. Originally the reabsorbed substances had been classified into two groups: those transferred because of passive back diffusion and the others – *Tran* substances – which are actively transferred by mechanisms of limited reabsorptive capacity. The subsequent investigations and interpretations are discussed which apparently have side tracked this original concept and led to a generally accepted theory of the renal handling of salt and water. It is emphasized that this theory is based on five interrelated assumptions which lack either direct experimental confirmation or have been disproved by direct observations. Thus it has not been established by any direct evidence, that the clearance of inulin ( *GFR* ) is determined exclusively by glomerular vascular factors (Assumpt I) nor has it been directly demonstrated that the rate of proximal sodium reab

sorption is dependent on the filtered load per se (Assumpt II) An assumed rate limiting concentration gradient for sodium in the proximal tubules (Assumpt III) does not exist, and proximal urea reabsorption proceeds *pari passu* with the reabsorption of sodium and water and therefore cannot contribute to an establishment of such gradient (Assumpt IV) Finally it has been demonstrated indirectly as well as directly that the hydrostatic pressure in the space of Bowman's capsule is not a constant parameter (Assumpt V) but this pressure varies as a primary and immediate event parallel with changes in the glomerular propulsive pressure

*The second chapter* is concerned with transcellular transport processes it is attempted to characterize the reabsorption processes in the mammalian proximal tubules at the cellular level The generally accepted model for active transcellular sodium transport is outlined Gradient limitation and  $T_m$  limitation of active transfer processes in general are discussed and examples are given to show that active sodium transport across certain amphibian epithelia (frog skin and toad bladder) may be  $T_m$  limited Across these latter epithelia the entry of sodium into the cell from the morphological outside solution appears to be the rate limiting step in the transcellular transfer process Whether or not this step in the transfer also has a rate limiting effect on the reabsorption of salt in the proximal tubules of mammalian nephrons remains to be established A review is given of micropuncture studies, designed in order to characterize the transport of each individual of the predominant ion species of the ultrafiltrate across each of the two cell borders of the mammalian proximal tubular cell, viz the luminal and peritubular membrane The interpretation of these results according to the underlying model seems to be that sodium is not merely 'pumped' actively out of the cell at the peritubular side it is also pumped into the lumen from the cell interior Also chloride ions apparently have to overcome a considerable electrochemical potential before they enter the cell at the luminal side and at this membrane potassium is actively 'pumped' into the cell Other direct investigations are discussed in which the relative rates of proximal net transfer of the main constituents of the ultrafiltrate are compared Notwithstanding the differently directed and unequally sized electrochemical forces created by the active ion transports it has emerged that potassium, chloride bicarbonate and urea are reabsorbed along the convoluted segment together with sodium and water in the same proportion as the one seen in the luminal fluid moreover a change in the rate of sodium reabsorption is accompanied by a parallel change in the rate of transfer of all the other mentioned constituents Thus the stereotyped proximal fluid reabsorption is seen in man to have the character of a bulk

reabsorption of an aqueous solution of electrolytes and urea. Studies on the water permeability of the proximal tubular wall indicated that the permeability is too low to account for the actually occurring water reabsorption as being due to osmosis exclusively, finally, estimations of a very low intracellular sodium concentration throw serious doubt on the generally accepted concept that transcellular transferred sodium is pumped across the peritubular membrane from a homogenous cytoplasmatic sodium pool. On the basis of this analysis the transcellular transport in the mammalian proximal tubules is suggested to take place in two steps: a normally rate limiting luminal 'micro pinocytosis' and a basal (peritubular) 'energy requiring mechanism of some yet undetermined, character'. In this hypothesis the transcellular transport process is spatially separated from the active transport processes which are responsible for the maintenance of the cytoplasmatic ion composition. The kinetics of such hypothetical, transcellular transport mechanism and its bearing on the interpretation of certain experimental data are discussed. It is concluded that a characterization of the reabsorption rate of salt and water as a  $T_m$  limited reabsorption is quite compatible with the mechanisms of transcellular transport in mammalian proximal tubules, as outlined in the proposed hypothesis.

*The third chapter* is concerned with the crucial question whether the proximal reabsorption rate of salt and water is load dependent or  $T_m$  limited. Previously proposed models of load dependent mechanisms for proximal salt reabsorption are mentioned and criticized, it is emphasized that the measured data upon which the models are based, directly indicate that the reabsorption rate is of a  $T_m$  limited character although they have been interpreted differently by the authors. Data obtained by the indirect clearance method are reviewed: it is concluded that the three types of investigations (salt loadings, induction of mechanical and dilution diureses) all are compatible with and in the case of dilution diuresis strongly support the concept of  $T_m$  limitation of the proximal reabsorption rate. A direct method by which to estimate the proximal reabsorption rate viz. the occlusion time method which has been applied to solve the problem in question is discussed in some detail. The occlusion time is defined as the time interval between the interruption of the renal circulation and filtration until the proximal tubules are emptied because of completed reabsorption of the luminal fluid. It is concluded that the method is a usable although rather rough method for an estimation of the proximal reabsorption rate. Data obtained by this method have indicated directly that the proximal reabsorption rate is independent of the filtered load supplied to the cells locally as well as downwards along



the entire convoluted segment of the proximal tubule. Thus, the data obtained by indirect as well as by direct methods have unequivocally demonstrated the  $T_m$  limited character of the proximal reabsorption rate of salt and water.

The conclusion that the proximal reabsorption rate is  $T_m$  limited involves two main consequences which are discussed in *chapter IV*.

First, hydrodynamic considerations on the pressure drop across the glomerular membrane and across the nephron will show that any change in the glomerular propulsive pressure (filtration pressure) will be accompanied primarily by a parallel change in the proximal intratubular pressure (and capsular pressure), which also is in accordance with indirect and direct evidence. Any changes in the effective filtration pressure, and therewith in the filtration rate, will not occur until the rate of proximal reabsorption has changed. This fact actually means that the rate of filtration is determined by the rate of proximal reabsorption. The implications of this realization for interpretations of clearance data are discussed. It further implies some rigidity of the proximal tubular wall, which must be able to resist a certain pressure difference between the tubular lumen and the surroundings. Otherwise any moderate changes in the intratubular pressure (and therewith in effective filtration pressure) induced by primary changes in the rate of reabsorption could not be established. Findings, by which such rigidity of the tubular wall is demonstrated, are discussed.

Secondly, the fact that the proximal rate of reabsorption does vary within a physiological range indicates that the limiting  $T_m$  is variable and a factor had to be looked for which might be responsible for these changes. Using the occlusion time method, angiotensin, which is released from the juxtaglomerular apparatus, was found to have such direct tubular effect, by which the proximal reabsorption rate is immediately and reversibly inhibited. Angiotensin was found to have a characteristic maximum tubular effect by which the reabsorption rate is depressed to the lowest physiological values. On the basis of the dose response relationship, a rough calculation will show that a change in the order of about 200 molecules per cell is sufficient to elicit a maximum physiological depression of the proximal reabsorption rate. The localization of the renin-angiotensin system to the juxtaglomerular apparatus together with the vascular and tubular actions of angiotensin, which readily explain the close correlation between the physiological range of variation in the renal blood flow, the proximal reabsorption rate and the rate of glomerular filtration, form the basis on which the conclusion is drawn that angiotensin is a physiological regulator of these parameters. Since the maximum tubular effect of angiotensin can be responsible for a depression of the

rate of filtration only to the lowest physiological values, other mechanisms are required by which to explain unphysiological (pathological or experimentally provoked) reductions in the clearance of inulin. In this survey data are included which demonstrate that such abnormal reductions in the inulin clearance are caused by the proximal luminal occlusion of ever increasing numbers of nephrons thus reducing the number of nephrons which participate in the urine formation.

*In the fifth chapter* conditions for a regulated salt excretion of the mammalian kidney is discussed in the light of the regulated and quickly variable limitation of the proximal reabsorption rate. It is argued that such regulated salt excretion rate, which is extremely critical for the maintenance of salt balance, and which might be seriously impaired by occurring vascular and metabolic changes unrelated with salt balance is conditioned by an efficient regulation of the proximal intratubular pressure which will maintain a fairly constant load of salt to the distal tubular segments. Data are reviewed which indicate that this pressure actually seems to be efficiently stabilized by some mechanism. The possibility is analysed that changes in the release of angiotensin may be responsible for this regulation. It is apparent from the literature that the release of renin or angiotensin generally is stimulated under conditions of decreased glomerular propulsive pressure probably by a stimulus acting from the tubular luminal side at the site of macula densa. Data obtained from one extreme experimental situation in which the arterial perfusion pressure to the isolated kidney is artificially elevated suggest indirectly however that the release of angiotensin may be increased also in this situation. The inference from such experiments is tentatively considered to suggest that the arteriolar vascular system may serve as a system of reference for normal stimuli to the juxtaglomerular apparatus acting from the tubular side. After a critical review of the previous hypotheses on the function of the juxtaglomerular apparatus the available data are comprised in a hypothesis viz that the physiological function of the juxtaglomerular apparatus is to establish a rapid intrarenal feed back mechanism whereby some function of a change in the proximal intratubular pressure controls the release of angiotensin thus involving that the proximal reabsorptive capacity and the efferent arteriolar resistance are adjusted in such a way that a stable proximal intratubular pressure is maintained. A resultant effect of this regulating mechanism is the maintenance of a fairly constant load of salt to the distal tubular segments irrespective of variations in the intrarenal turnover rate of salt and water provoked by stimuli unrelated to salt balance.

*In the last chapter (VI)* a few hitherto obscure observations are discussed which may be explained in the light of the proposed thesis. Thus in view of the hypothesis that a luminal micropinocytosis represents the rate limiting step in proximal fluid transfer angiotensin might be presumed to have a specific affinity to this mucopolysaccharide containing membrane this suggestion is not contradicted by the demonstrated, reversible affinity of angiotensin to heparin (a sulphuric ester of a polysaccharide). The lack of correlation between the glomerular filtration rate and the electrolyte excretion rates under various conditions is discussed and on the basis of the hypothesis advocated here it might be predicted but would seem unexplainable if considered on the basis of the previous concepts. Changes in the rates of electrolyte excretion renal blood flow and filtration rate induced by expansion of the extracellular volume are explained in accordance with the thesis and the well established stimulating effect of angiotensin on the adrenal aldosterone secretion. The qualitative similarity of the integrated renal functional responses to heavy doses of angiotensin and noradrenaline irrespective of the absence of any direct immediate tubular effect of noradrenaline is discussed according to the hypothesis this and other features observed concerning the renal effects of noradrenaline may be explained as secondary events mediated by rapid changes in the release of endogenous angiotensin. The necessity of a voluminous proximal fluid reabsorption characteristic of all mammalian nephrons is comprehensible when it is realized that a stable proximal intratubular pressure is a prerequisite of a regulated salt excretion rate controlled by a mechanism which has to operate not only by changing the post glomerular vascular resistance but also the proximal reabsorptive capacity. With a great capacity and range of variation relative to occurring changes in the glomerular propulsive pressure the possibility for an efficient pressure regulation has been created. It is mentioned finally that the proposed regulating mechanism permitting an efficient sodium conservation to operate independent of simultaneous water excretion seems equally necessary for homeothermic urea excreting animals adapted to terrestrial life as does an efficient water conserving mechanism. Thus it is in accordance with the present thesis that the juxtaglomerular apparatus containing the renin-angiotensin system to our knowledge is found only in birds and mammals and thus seems to have been evolved concurrently with and interdependent with the loop of Henle.

## Dansk resume

Det generelle biologiske krav om saltbalance i organismen som finder udtryk i existensen af et stabilt extracellulært vædskeum kan for pattedyrs vedkommende kun opfyldes ved en regulering af den renale saltudskillelse. For at kunne afsløre og forstå de mekanismer der betinger en sådan regulering af nyrens saltudskillelse har det været nødvendigt at udarbejde metoder som tillod en klar adskillelse mellem vasculære og tubulære faktors indflydelse på nyrens behandling af salt og vand. Denne oversigt beskæftiger sig overvejende med data opnaet i studier over filtratreabsorptionen i pattedyr nefronernes proximale tubuli hvor langt den største del af det filtrerede salt og vand reabsorberes. Dette emne for den eksperimentelle del af undersøgelsen hvorpå oversigten er baseret blev valgt fordi der ikke var almindelig enighed om hvorvidt reabsorptionshastigheden for salt og vand i dette tubulus afsnit er afhængig eller uafhængig af det filtrerede salttilbud. Denne oversigts afhandlings hovedtema er analysen af dette problemkompleks som har fundamental betydning for hele vor opfattelse af de mekanismer der ligger til grund for urindannelsen og især for de der betinger reguleringen af saltudskillelsen.

I første kapitel omtales kort nogle tidligere bidrag til forståelsen af hovedtrækkene i nyrens funktion. De reabsorberede stoffer synes oprindeligt at være blevet klassificeret i to grupper. Stoffer der transporteres ved passiv tilbage diffusion og de andre – *Tm* stoffer – der aktivt transporteres af mekanismer med begrænset transportkapacitet. De senere undersøgelser og tolkninger som øjensynligt har afsporet denne oprindelige opfattelse og ført til en generelt antaget teori for nyrens behandling af salt og vand bliver diskuteret. Det fremhæves at denne teori bygger på fem indbyrdes afhængige antagelser som enten mangler direkte eksperimentel støtte eller er modbevist af direkte tagtagelser. Det er således aldrig blevet vist direkte at *inulin* clearance ( *GFR* ) udelukkende er bestemt af de vasculære faktorer i glomeruli (I antagelse) ligesom det heller aldrig er blevet påvist direkte at reabsorptionshastigheden for natrium er afhængig af selve tilbuddet (II antagelse). Det er vist direkte at der ikke eksisterer nogen hastighedsbegrænsende koncentrationsgradient for natrium i de proximale tubuli (III antagelse) og at urinstof reabsorptionen her går parallelt med vand og saltreabsorptionen og derfor ikke kan bidrage

til tilvejebringelse af en sådan postuleret natrium gradient (IV' antagelse) Endelig er det blevet vist både indirekte og direkte at det hydrostatiske tryk i Bowman's kapselrum ikke er en konstant parameter (V' antagelse), men tværtimod primært og umiddelbart varierer parallelt med ændringer i det glomerulære propulsive tryk

*Det andet kapitel* omhandler transcellulære transportprocesser da formålet har været et forsøg på at karakterisere reabsorptionsprocesserne i pattedyr nyrens proximale tubuli på det cellulære niveau Den generelt antagne model for transcellulær natriumtransport skitseres Gradient limitation og Tm limitation for aktiv transport i almindelighed diskuteres og der gives eksempler på at aktiv natriumtransport over visse amphibieepithelier (froskind og tudseblære) kan være Tm limiteret I disse epithelier synes det at være natriums optagelse i cellen fra opløsningen på dens morfologiske udside, der er det limiterende trin for den transcellulære transportproces hastighed Det vides ikke om det også er dette trin, der er hastighedsbegrænsende for saltreabsorptionen i pattedyrnyfronernes proximale tubuli Der gøres rede for resultaterne af en række micropunkturundersøgelser, der har taget sigte på at karakterisere transporterne af natrium og ultrafiltratets andre ioner over hver af de to cellegrenser i pattedyrnyrens proximale tubulusceller, henholdsvis den lumenale og den peritubulære membran Ud fra den tilgrundliggende model opfattelse må disse resultater fortolkes således at natrium ikke direkte pumpes aktivt ud af cellen over den peritubulære membran, men også pumpes ind i lumen fra cellen Klor ionerne må tilsyneladende også overvinde et betydeligt elektrochemisk potentiale for at komme ind i cellen fra lumenvædsken og over den lumenale membran pumpes kalium aktivt ind i cellen Der omtales en række andre undersøgelser som tillader en sammenligning af de relative netto transporthastigheder i de proximale tubuli af ultrafiltratets hovedbestanddele Det har vist sig at kalium, klor bicarbonat og urinstof reabsorberes sammen med natrium og vand ned igennem det proximale slyngede segment i det samme forhold som findes i tubulusvædsken til trods for de forskelligt rettede og uens store elektrochemiske kræfter, der er skabt af de aktive ion transporter og ydermere at en ændring i natrium reabsorptionshastigheden vil ledsages af en parallel ændring i transporthastigheden af alle de nævnte bestanddele af filtratet Den noget stereotype proximale vand reabsorption fremtræder således i det væsentlige som en bulk reabsorption af en vandig opløsning af elektrolyter og urinstof Undersøgelser over den proximale tubulusvægs vand permeabilitet har vist at permeabiliteten er for lille til at kunne forklare den faktisk forekommende vandreabsorption som ren osmose og endelig har det været muligt at skønne at den intracellulære natrium concentration er så

ringe at det i høj grad må betvivles at transcellulært transporteret natrium kan pumpes over den peritubulære membran fra en homogen cytoplasmatisk natrium pool

På basis af denne analyse fremsættes hypotesen, at den transcellulære transport i pattedyrnyrens proximale tubuli sker i to trin. En under normale forhold hastighedsbegrænsende luminal 'micropinocytose' og en basal (peritubulær), energikrævende mekanisme af en eller anden art. Ud fra denne hypotese er den transcellulære transportproces rumligt adskilt fra de aktive transportprocesser, der opretholder cytoplasmaets normale ion sammensætning. Kinetiken for en sådan hypotetisk transcellulær transportmekanisme og dens relation til tolkningen af visse forsøgsresultater bliver diskuteret. Det konkluderes at muligheden for Tm-limitering af hastigheden for salt og vandreabsorptionen i pattedyrnyrens proximale tubuli er helt forenelig med opfattelsen af den transcellulære transportmekanisme således som den er skitseret i den fremsatte hypotese. Endelig gives der en kort omtale af den proximale glukosereabsorption set i lyset af denne hypotese.

*Det tredje kapitel* omhandler det afgørende spørgsmål om hvorvidt den proximale reabsorptionshastighed for salt og vand er, loadafhængig eller Tm-limiteret. Tidligere fremsatte modeller til forklaring af loadafhængige mekanismer for den proximale saltreabsorption nævnes og kritiseres og det fremhæves at målingerne på hvilke modellerne er baseret, faktisk direkte viser, at reabsorptionshastigheden må være Tm-limiteret, skønt forfatterne tolker resultaterne anderledes. Der gennemgås resultater opnået med den indirekte clearance metode og det sluttes at de tre forsøgstyper (salt belastning mekaniske diureser og fortyndingsdiurese) alle har givet resultater der er forenelige med og for fortyndingsdiuresens vedkommende stærk støtter opfattelsen af at den proximale reabsorptionshastighed er Tm-limiteret. En direkte metode til vurdering af den proximale reabsorptionshastighed occlusionstids metoden som har været brugt til at løse det pågældende problem diskuteres indgående. Occlusionstiden er defineret ved den tid der medgår fra afbrydelse af nyrens kredsløb og filtration indtil de proximale tubuli har tømt sig for luminal vædske ved den fortsatte reabsorption. Det sluttes at denne metode er en anvendelig omend temmelig grov metode til vurdering af den proximale reabsorptionshastighed. Resultater opnået med denne metode har direkte vist at den proximale reabsorptionshastighed er uafhængig af det filtrerede tilbud til cellerne lokalt såvel som ned langs hele det slængede segment af den proximale tubulus. Således har målingerne både med indirekte og direkte metoder utvetydigt vist den Tm-limiterede karakter af den proximale reabsorptionshastighed for salt og vand.

De to væsentligste konsekvenser af konklusionen At den proximale reabsorptionshastighed er  $T_m$  limiteret – diskuteres i *fjerde kapitel*

For det første vil hydrodynamiske overvejelser over trykfaldene over glomerulusmembranen og over nefronet vise, at enhver ændring i det propulsive tryk (filtrationstrykket) primært vil ledsages af en parallel ændring i det proximale intratubulære tryk (og kapseltrykket) i overensstemmelse med indirekte og direkte opnaede måleresultater Der vil således ikke forekomme ændringer i det effektive filtrationstryk og dermed i filtrationshastigheden forend den proximale reabsorptionshastighed ændres hvilket faktisk betyder at filtrationshastigheden er bestemt af hastigheden af den proximale reabsorption Betydningen af denne erkendelse for tolkningen af clearance resultater bliver diskuteret Det indebærer nderligere at der må være en vis stivhed af den proximale tubulusvæg, som må være i stand til at modstå nogen trykforskel mellem lumen og omgivelserne I modsat fald ville selv beskedne ændringer i det intratubulære tryk (og dermed i det effektive filtrationstryk) fremkaldt ved primære ændringer i reabsorptionshastigheden ikke kunne opstå Der omtales målinger der pviser en sådan stivhed af tubulusvæggen

For det andet viser den kendsgerning at den proximale reabsorptionshastighed varierer inden for et fysiologisk område at den hastighedsbegrænsende  $T_m$  er variabel hvorfor man måtte søge efter en faktor der kunne tænkes at være ansvarlig for de forekommende ændringer i den proximale reabsorptionskapacitet inden for dette område Det er blevet vist med occlusionstids metoden at angiotensin der frigøres fra det juxtaglomerulære apparat har en sådan direkte tubulær virkning hvorved den proximale reabsorptionshastighed hæmmes øjeblikkeligt og reversibelt Der er påvist en karakteristisk maximal tubulær angiotensineffekt hvorved reabsorptionshastigheden bliver reduceret til den laveste værdi der ses spontant Et groft skøn baseret på dosis response afhængigheden viser at en ændring på omkring et par hundrede molekyler angiotensin per celle er i stand til at udløse en maximal fysiologisk hæmning af den proximale reabsorptionshastighed Lokaliseringen af renin angiotensin systemet til det juxtaglomerulære apparat og den vasculære og tubulære virkning af angiotensin som utvungen forklarer den nøje korrelation mellem og det fysiologiske variationsområde af nyrens gennemblødning den proximale reabsorptionshastighed og filtrationshastigheden er de præmisser der fører til konklusionen at angiotensin er en fysiologisk regulator af disse parametre Da den maximale tubulære angiotensineffekt kun kan være ansvarlig for et fald i filtrationshastigheden ned til det laveste område der ses fysiologisk må der være andre mekanismer der er ansvarlige for ufysiologiske (pathologiske eller eksperimentelt fremkaldte) fald i mulin clearance Der omtales under søgelser der har vist at sådanne abnorme fald i mulin clearance skyldes occlu

sion af proximale lumina af flere og flere nefroner hvorved antallet af nefroner der deltager i urindannelsen aftager

*I femte kapitel* diskuteres forudsætningerne for at have en reguleret saltudskillelse i pattedyrnyren set udfra den situation at der er en reguleret og hastigt variabel  $\text{Tm}$  limiteret proximal reabsorptionshastighed. Det pointeres at betingelsen for en sådan reguleret saltudskilleleshastighed som må være overordentlig kritisk for opretholdelsen af saltbalancen og som i høj grad vil kunne trues af forekommende vasculære og metaboliske ændringer der i sig selv ikke har nogen relation til saltomsætningen må være en effektiv regulering af det proximale intratubulære tryk hvorved et ret konstant salttilbud til de distale tubulusnit vil kunne opretholdes. Der gennemgås resultater som viser at dette tryk faktisk forekommer at være effektivt stabiliseret af en endnu ukendt mekanisme og den mulighed at ændringer i angiotensinfrigørelsen kunne være ansvarlig for denne regulering analyseres. Det fremgår af litteraturen at renin eller angiotensinfrigørelsen sædvanligvis stimuleres under forhold hvor det glomerulære propulsive tryk tenderer til at falde sandsynligvis forårsaget af et stimulus der virker fra den tubulært lumenale side ud for macula densa. Imidlertid lader resultater opnået i en ekstrem experimentel situation hvor det arterielle perfusionstryk til den isolerede nyre kunstigt hæves indirekte formode at angiotensinfrigørelsen også i denne situation kan være øget. En sådan formodet frigørelse i denne situation kan muligvis tages som udtryk for at arteriolerne tjener som et referencesystem for normale stimuli til det juxtaglomerulære apparat fra tubulus-siden. Efter en kritisk gennemgang af tidligere hypoteser om det juxtaglomerulære apparats funktion forsøger man at indordne tilgængelige data i følgende hypotese: At det juxtaglomerulære apparats fysiologiske funktion er at etablere en feed back mekanisme hvorved visse ændringer der opstår som en ikke nøjere defineret funktion af ændringer i det proximale intratubulære tryk regulerer angiotensinfrigørelsen således at den proximale reabsorptionskapacitet og den efferente arteriolemodstand tilpasses efter forholdene på en sådan måde at det proximale intratubulære tryk holdes stabilt. En sådan reguleringsmekanismes effekt vil være at vedligeholde et temmelig konstant salttilbud til de distale tubulusafsnit uafhængig af ændringer i den intrarenale omsætningshastighed af salt og vand der måtte være fremkaldt af stimuli der er uden relation til organismens saltbalance.

*I det sidste kapitel (VI)* forsøges nogle enkelte tidligere uforklarlige iagttagelser forklaret ud fra den fremsatte these. Ud fra den opfattelse at det hastighedsbegrænsende trin i den proximale vædske-transport er en luminal



micropinocytose skulle man forvente en ret specifik affinitet af angiotensin til den luminal membran der indeholder betydelige mængder mucopolysaccharid, en formodning der kunne passe med en påvist reversibel affinitet af angiotensin til heparin (en svovlsyreester af et polysaccharid). Den manglende sammenhæng mellem filtrationshastigheden og electrolyt udskillelseshastigheden under en række tilstande er forudsigelig ud fra den fremsatte hypotese men uforståelig i lyset af tidligere opfattelser. De ændringer, der fremkommer i electrolytudskelelseshastighederne nyrrens gennemblodning og i filtrationshastigheden ved expansion af det extracellulære vædskerum, forklares i overensstemmelse med thesen og det fastslåede forhold, at angiotensin stimulerer binyrernes aldosteronsekretion. Den kvalitative lighed imellem de integrerede nyrefunktionsændringer der fremkommer under indflydelse af kraftigt virkende doser angiotensin og noradrenalin på trods af den kendsgerning at noradrenalin ikke har nogen øjeblikkelig direkte tubulær virkning bliver omtalt, og denne og andre iagttagelser over noradrenalins renale virkninger forklares i overensstemmelse med hypotesen som sekundære effekter udløst ved hurtige ændringer i frigørelsen af endogent angiotensin. Når det erkendes, at en forudsætning for en reguleret saltudskillelse er et stabilt proximalt, intratubulært tryk der kontrolleres af en mekanisme som nødvendigvis må virke ikke blot ved ændringer i den post glomerulære karmodstand, men også i høj grad ved ændringer i den proximale reabsorptionskapacitet, bliver nødvendigheden af en så voluminøs proximal vædskereabsorption som er karakteristisk for alle pattedyr nefroner også forståelig. Med stor kapacitet og et stort variationsområde i forhold til de muligt forekommende ændringer i det glomerulære propulsive tryk er der skabt muligheder for en effektiv trykregulering. Til sidst nævnes det at en sådan reguleringsmekanisme der gør det muligt for organismen at konservere natrium effektivt og uafhængigt af samtidig vandudskillelse må være lige så nødvendig som en effektiv vandkonserverende mekanisme for varmblodige, urinstofudskillende dyr, der er adapteret til et liv på landjorden. Det synes derfor at være foreneligt med den fremsatte these at et juxta glomerulært apparat med renin angiotensin systemet, såvidt vides kun forekommer hos fugle og pattedyr hvor det således synes udviklet i indbyrdes afhængighed af og samtidigt med udviklingen af Henle's sløjnge

## Acknowledgement

The present investigation was carried out in the years between 1960 and 1964 at the University Institute for Experimental Medicine in Copenhagen where I have had literally ideal conditions for research work. I am greatly indebted to and want to thank the director of the institute dr med Jens Bing for his unfailing stimulation to my work which was started in an attempt to contribute to the disclosure of the unknown physiological significance of the juxtaglomerular apparatus. Dr Bing's most valuable criticism and his highly sober attitude to research, as expressed in many interesting discussions, has been an essential part of my introduction to a scientific way of thinking and education and it has been a great experience to have had the opportunity of working as one of his collaborators. I also thank Dr Bing heartily for his valuable help in obtaining financial support to this investigation.

I thank professor dr med Poul Kruhoffer for his most valuable criticism and many helpful suggestions during my work and in preparing the manuscripts to the original papers. His clear logic and sense of the details to a clear formulation and description has been highly instructive.

I also want to thank professor dr med K. Brochner Mortensen for his stimulating interest in my work during the tenure of my research fellowship. Professor Carl W. Gottschalk M.D. of the University of North Carolina Chapel Hill, N.C. gave me much valuable instruction and advice to the micropuncture technique during his stay in Copenhagen and I have had much benefit of our many interesting discussions.

Lecturer mag. scient. B. Svejgaard Nielsen from the Mathematical Institute of the University of Copenhagen has excellently contributed to the solution of efflux kinetics in isolated tissue cylinders which was a most essential step to the interpretation of data obtained in studies on transcellular transport processes performed in collaboration with dr med Ejgil Bojesen.

Lecturer mag. scient. Hans Brøns from the Institute of Mathematical Statistics of the University of Copenhagen has very kindly helped me with the statistical treatment of the dose response relationship of angiotensin and contributed to the formulation of a usable statistical model and cand. stat. Inger Harder Hansen has skilfully carried out the calculations and given many good suggestions for which I express my gratitude.

During my work I have had much help from and stimulating discussions with dr Ejvind Kemp Jørn Giese Poul Faarup, Henrik Olesen, mag scient Poul K. Jensen and veterinary J Kazimierzak, who all contributed also to the pleasant atmosphere of the Institute

I am especially grateful to my skilful technical assistant miss Anni Hutter who has helped me brilliantly and always has been interested and encouraging to my experimental work I also thank photographer Ulla Eberth for her help and advice with microphotography in vivo and for her manufacture of figures for the manuscripts The mechanical engineer of the Institute H Skjold Petersen, has manufactured with great skill a series of special instruments and apparatus for my investigations, for which I thank him heartily

Mrs Agnete Lund has perfectly typewritten the fair copies of the manuscripts to the original papers and translator, mrs A Engelbreth Holm has corrected my English language I thank both for their excellent contributions

I want to express my sincere thank to the whole staff of the Institute and to the many visitors, Danish as well as foreign, who all have been generously helpful in many ways

The present investigation was financially supported by grants from Statens almindelige Videnskabs Fond Novofonden and Kong Christian den Tiendes Fond Moreover support for the purchase of special apparatus was generously supplied by Carlsberg Fonden and P Carl Petersens Fond The well scintillation counter and scaler used for efflux studies of sodium in vitro was generously supplied by Tuborg Fonden I sincerely want to thank for this support which has been most essential for the accomplishment of the present investigation

## References

- Barajas I and H Latta The juxtaglomerular apparatus in adrenalectomized rats *Lab Invest* 1963 17 1046-1059 (76 107)
- Bean J W Specificity in the renin hypertensinogen reaction *Amer J Physiol* 1947 136 731-742 (117)
- Biber Th. U L M Mylle and C W Gottschalk, Micropuncture study of kidney function in rats with experimental tubular necrosis *Proc II int Congr Nephrol Prague* 1963 *Excerpta Med Found Amsterdam* 1964 21-27 (17)
- Bing J Angiotensinase content of kidney of normal and hypertensive rats *Acta path microbiol scand* 1962 a 56 385-387 (92)
- Bing J Relation between renal renin content and hypertension I: Renal renin content in normal unilaterally nephrectomized and renal hypertensive rats *Scand J clin lab Invest* 1962 b 14 15-26 (93)
- Bing J Morphological aspects of the renin angiotensin system *Danish Med Bull* 1964 2 24-33 (77 108)
- Bing J and J Kazimierzak Renin content of different parts of the periglomerular circumference *Acta path microbiol scand* 1960 50 1-11 (76)
- Bing J and J Kazimierzak Renin content of different parts of the juxtaglomerular apparatus *Acta path microbiol scand* 1962 54 80-84 (76)
- Bing J and J Kazimierzak Renin in nephrogenic renal tissue devoid of both granular and non granular epitheloid juxtaglomerular cells *Acta path microbiol scand* 1964 60 83-89 (77)
- Bing J and B Wiberg Localisation of renin in the kidney *Acta path microbiol scand* 1958 44 138-145 (76)
- Blair West J R J P Coghlen D A Denton J R Goding J A Munro R F Peterson and M Wintour Humoral stimulation of adrenal cortical secretion *J clin Invest* 1962 41 1606-1625 (113 114)
- Blake W D R Wegria H P Ward and C W Frank Effect of renal artery constriction on excretion of sodium and water *Amer J Physiol* 1950 163 422-429 (21 58)
- Bliss E L ■ Rubin and T Gilbert The effect of adrenaline on adrenal cortical function *J clin Endocrinol* 1951 11 46-60 (116)
- Bojesen E The transport of urine in the upper urinary tract *Acta physiol scand* 1954 a 32 39-62 (24 57)
- Bojesen E The renal mechanism of dilution diuresis and salt excretion in dogs *Acta physiol scand* 1954 b 32 129-147 (24 52 57 59 71 75 77 113 114)
- Bojesen E *Den tubulare salt- og vandresorption* Christens Bogtrykkeri København 1955 Thesis pp 67 (57 77)
- Bojesen E The dependence of the glomerular filtration rate of water on glomerular and tubular factors In *Nierenfunktion und Nervensystem Int Symp VEB Verlag Volk und Gesundheit Berlin* 1957 185-189 (77)
- Bojesen ■ and H Degn Influence of changes of blood volume on the concentration of aldosterone in peripheral plasma of intact unanaesthetized dogs *Nature* 1961 190 352-353 (114)
- Bojesen F and P F Leyssac The kidney cortex slice technique as a model for sodium transport in vivo A qualitative evaluation *Acta physiol scand* 1965 65 20-32 (35 37 43 44 45)

- Bojesen E P P Leyssac and B Svejgaard Nielsen Analysis of the efflux kinetics of sodium from small cylinders of rabbit kidney cortex *Acta physiol scand* 1965 65 105-119 (44)
- Bonting S L M R Canady and N M Hawkins Angiotensin and renal Na K activated adenosine triphosphatase Studies on Na K activated ATP ase VIII *Biochim Biophys Acta* 1964 82 427-429 (112)
- Boucher R R Veyrat J de Champlain and J Genest New procedure for measurement of human plasma angiotensin and renin activity levels *Canad Med Ass J* 1964 90 194-201 (96)
- Brown J J D L Davies A F Lever R A Parker and J I S Robertson Assay of renin in single glomeruli Renin distribution in the normal rabbit kidney *Lancet* 1963 a II 668-670 (96 99)
- Brown J J D L Davies A F Lever and J I S Robertson Influence of sodium loading and sodium depletion on plasma renin in man *Lancet* 1963 b II 278-279 (91)
- Brown J J D L Davies A F Lever and J I S Robertson Variations in plasma renin concentration in several physiological and pathological states *Canad Med Ass J* 1964 a 90 201-206 (96)
- Brown J J D L Davies A F Lever and J I S Robertson Influence of sodium deprivation and loading on the plasma renin in man *J Physiol (Lond)* 1964 b 173 408-419 (99)
- Chapman Andresen C Studies on pinocytosis in amoebae *Compt rend trav Lab Carlsberg* 1962 33 73-264 Thesis (111)
- Chapman Andresen C and H Holter Differential uptake of protein and glucose by pinocytosis in Amoeba Proteus *Compt rend trav Lab Carlsberg* 1964 34 211-226 (48 111)
- Clapp J R J F Watson and R W Berliner Osmolality bicarbonate concentration and water reabsorption in proximal tubule of the dog nephron *Amer J Physiol* 1963 205 273-280 (41 109)
- Cook W F D B Gordon and W S Peart The location of renin in the rabbit kidney *J Physiol (Lond)* 1956 135 46 P (76)
- Cook W F and G W Pickering The location of renin in the rabbit kidney *J Physiol (Lond)* 1959 149 526-536 (76)
- Cuthny A R *The secretion of urine* Ed E H Starling Longmanns Green and Co London 1917 I Ed pp 241 (13)
- Davis J O Aldosterone and angiotensin *J Amer Med Ass* 1964 189 1062-1068 (113)
- Davis J O C C J Carpenter R C Ayers J E Holman and R C Bahn Evidence for secretion of an aldosterone stimulating hormone by the kidney *J clin Invest* 1961 40 684-696 (113)
- Deetjen P and K Kramer Na Rückresorption und O Verbrauch der Niere *Klin Wchsch* 1960 38 680 (45)
- Duncan L II jr D H Solomon M P Nichols and E Rosenberg The effect of the chronic administration of adrenal medullary hormones to man on adrenocortical function and the renal excretion of electrolytes *J clin Invest* 1951 30 908-915 (116)
- Edelman R and Ph M Hartroft Localization of renin in juxtaglomerular cells of rabbit and dog through the use of the fluorescent antibody technique *Circul Res* 1961 II 1069-1077 (77)
- Egler F W Short circuit current measurements in proximal tubule of Necturus kidney *Amer J Physiol* 1961 201 157-163 (20 36)

- Faarup P The morphology of the juxtaglomerular apparatus *Proc II int Congr Nephrol Prague 1963 Excerpta Med Found Amsterdam 1964* 474-477 (75 107)
- Faarup P and H E Christensen Substructures in basement membranes of the renal medulla studied in freeze dried tissue *Acta path microbiol scand 1965* 64 71-82 (71)
- Farquhart M G ■ I Wissig and G E Palade Glomerular permeability I Ferritin transfer across the normal glomerular capillary wall. *J exper Med 1961* 113 47-65 (71)
- Fetterman G H N A Shuplock F J Philipp and H S Gregg The postnatal growth and maturation of the glomeruli and proximal convolutions in the human kidney with a note on cortical nephrons Studies by microdissection *Proc II int Congr Nephrol Prague 1963 Excerpta Med Found Amsterdam 1964* 37-38 (64)
- Folkow ■ B Johansson and E Mellander The comparative effects of angiotensin and noradrenaline on consecutive vascular sections *Acta physiol scand 1961* 53 99-105 (105)
- Frazier H S E F Dempsey and A Leaf Movement of sodium across the mucosal surface of the isolated toad bladder and its modification by vasopressin *J gen Physiol 1962* 45 529-543 (33 79)
- Genest J R Boucher J de Champlain R Veyrat M Chretien P Biron G Tremblay P Roy and P Carter Studies on the renin-angiotensin system in hypertensive patients *Canad Med Ass J 1964* 90 263-268 (96)
- Genest J W Nowaczynski E Kow T Sandor and P Biron *Adrenocortical Function in Essential Hypertension* In *Essential Hypertension* Int Symp Edd K D Bock and P T Cottier Springer Verlag Berlin 1960 pp 39' 126-146 (113)
- Gertz K H Direct measurement of the transtubular flux of electrolytes and non electrolytes in the intact rat kidney *Proc int Union Physiol Sci 27 int Congr Leiden 1962* I (1) 370-371 (78)
- Gertz K H Transubuläre Natriumchloridflüsse und Permeabilität für Nichteletrolyte im proximalen und distalen Konvolut der Rattenniere *Pflugers Arch ges Physiol 1963* 276 336-356 (36 52 64 70)
- Gertz K H G C Kennedy and K J Ullrich Mikropunktionsuntersuchungen über die Flüssigkeitdruckresorption aus den einzelnen Tubulusabschnitten bei Wasserdiurese (Diabetes Insipidus) *Pflugers Arch ges Physiol 1964* 278 513-519 (92 109)
- Giebisch G Electrical potential measurements on angle nephrons of *Necturus* *J Cell comp Physiol 1958* 51 221-239 (20)
- Giebisch G R M Klose and E E Windhager Micropuncture study of hypertonic sodium chloride loading in the rat *Amer J Physiol 1964* 206 687-693 (21 49 55 88)
- Giebisch G and E E Windhager Renal tubular transfer of sodium chloride and potassium *Amer J Med 1964* 36 643-669 (35 38)
- Goldblatt H J Lynch R F Hanzal and W W Summerville Studies on experimental hypertension I The production of persistent elevation of systolic blood pressure by means of renal ischemia *J exper Med 1934* 59 347-379 (92)
- Goormaghtigh N Histological changes in the ischemic kidney With special reference to the juxtaglomerular apparatus *Amer J Path 1940* 16 409-416 (75 76 98)
- Gottschalk C W Micropuncture measurements of intrarenal pressures *Circul Res 1964 a Suppl 1* 15 110-114 (75)
- Gottschalk C W Osmotic concentration and dilution of the urine *Amer J Med 1964 b* 36 679-685 (39)
- Gottschalk C W and M Mylle Micropuncture study of pressures in proximal tubules and peritubular capillaries of the rat kidney and their relation to ureteral and renal venous pressures *Amer J Physiol 1956* 185 430-439 (23 25 53 71 73 74 88 102)

- Gottschalk C W and M Mylle Micropuncture study of pressures in proximal and distal tubules and peritubular capillaries of the rat kidney during osmotic diuresis *Amer J Physiol* 1957 189 323-329 (23 73)
- Gross F and F Sulzer Der Einfluss der Nebennieren auf die blutdrucksteigernde Wirkung von Renin und auf pressorische Substanzen in den Nieren *Arch exper Path Pharmacol* 1957 230 274-283 (93)
- Guyton A C J II Langston and G Navar Theory for renal autoregulation by feed back at the juxtaglomerular apparatus *Circ Res* 1964 15 187-196 (105)
- Haas E and H Goldblatt Renin content of kidney in experimental renal and human essential hypertension *Amer J Physiol* 1959 197 1103-1106 (93)
- Hanussen O E Early post mortem renal changes studied in mice with one kidney extenized II The functional and the early post mortem morphology of the kidney *Acta path microbiol scand* 1960 49 297-320 (61)
- Hanussen O F and L Hermin The presence of an axial structure in the microvillus of the mouse convoluted proximal tubular cell *Lab Invest* 1962 11 610-616 (45)
- Hartroft Ph M L E Sutherland and W S Hartroft Juxtaglomerular cells as the source of renin Further studies with the fluorescent antibody technique and the effect of passive transfer of antirenin *Canad Med Ass J* 1964 90 163-166 (93)
- Hays R M and A Leaf Studies on the movement of water through the isolated toad bladder and its modification by vasopressin *J gen Physiol* 1962 45 905-919 (78)
- Higgins J T J O Davis and J Urquhart Demonstration by pressor and steroidogenic assays of increased renin in lymph of dog with secondary hyperaldosteronism *Circ Res* 1964 14 218-227 (94)
- Higgins J T J O Davis J Urquhart and M J Olchney Increased plasma level of renin in experimental secondary hyperaldosteronism *Amer J Physiol* 1964 207 814-820 (94)
- Hinshaw L B Mechanism of autoregulation Role of tissue pressure and description of a multifactor hypothesis *Circ Res* 1964 Suppl 1 15 120-129 (25 108)
- Hudobro F and E Braun Menendez The secretion of renin by the intact kidney *Amer J Physiol* 1942 137 47-53 (95)
- Juques R, K Kuttner H J Bein and R Meier Bindung eines synthetischen Poly peptides (Hypertensin) an Heparin und Freisetzung des Peptides durch Component 48/80 in vitro *Experientia* 1960 16 147-149 (111)
- Kamm D E and N G Levinsky Effect of plasma sodium elevation on renal sodium reabsorption *Amer J Physiol* 1964 206 1131-1136 (57)
- Kaplan N M and F C Barter The effect of ACTH renin angiotensin II and various precursors on biosynthesis of aldosterone by adrenal slices *J clin Invest* 1962 41 715-724 (113)
- Kaplan A and M Friedman Studies concerning the site of renin formation in the kidney III The apparent site of renin formation in the tubules of the mesonephros and metanephros of the hog fetus *J exper Med* 1949 76 307-316 (77)
- Kashgarian M H Stöckle C W Gottschalk and K J Ullrich Transtubular electrochemical potentials of sodium and chloride in proximal and distal renal tubules of rats during antidiuresis and water diuresis (Diabetes insipidus) *Pflugers Arch ges Physiol* 1963 277 89-106 (36 37 38 40)
- Kelman R B A theoretical note on exponential flow in the proximal part of the mammalian nephron *Bull Math Biophys* 1962 24 303-317 (54)
- Kemp E Destruction and liberation of enzymes in the kidney of normal and hypertensive rabbits *Acta path microbiol scand* 1960 48 186-196 (94)
- Kemp E *Nyreens ymstudier Med særligt henblik pa reninsystemet Aarhus Stiftsbog trykkeri København* 1962 pp 100 The is (92)

- Kemp E and T Laursen On the renal release and excretion of enzymes in experimental nephritis and shock *Acta path microbiol scand* 1962 54 85-95 (94)
- Koefoed Johnsen V and H H Ussing The contributions of diffusion and flow to the passage of D<sub>2</sub>O through living membranes *Acta physiol scand* 1953 28 60-76 (78)
- Koefoed Johnsen V and H H Ussing The nature of the frog skin potential *Acta physiol scand* 1958 42 298-308 (39)
- Kruhoffer P *Studies on water electrolyte excretion and glomerular activity in the mammalian kidney* Rosenkilde and Bigger Copenhagen 1950 pp 241 Thesis (19 49 50 56 86)
- Kruhoffer P *IV Handling of the alkali metal ions by the kidney* In Ussing H H P Kruhoffer J H Thaysen and N A Thorn The Alkali Metal Ions in Biology Springer Verlag Heidelberg 1960 233-432 (19 23 57 60 70 115)
- Kuhn W *Mechanismus der Harnkonzentrierung der Niere* In Glomerulare und tubuläre Nierenerkrankungen Int Symp Georg Thieme Verlag Stuttgart 1967 3-17 (119)
- Langford H G Tubular action of angiotensin *Canad Med Ass J* 1964 90 332-333 (83)
- Langford H G and N E Fallis Tubular effect of angiotensin *Circulation* 1963 28 754 (83)
- Langston J B, A G Guyton J H dePoyster and G C Armstrong Changes in renal function resulting from norepinephrine infusions *Amer J Physiol* 1962 202 893-896 (115)
- Laragh J H M Angers W K Kelly and S Lieberman Hypotensive agents and pressor substances The effect of epinephrine norepinephrine angiotensin II and others on the secretory rate of aldosterone in man *J Amer Med Ass* 1960 a 174 234-240 (113)
- Laragh J H P J Cannon and R P Ames Interaction between aldosterone secretion sodium and potassium balance and angiotensin activity in man Studies in hypertension and cirrhosis *Canad Med Ass J* 1964 90 248-256 (113)
- Laragh J H S Ulick V Januszewicz W G Kelly and S Lieberman Electrolyte metabolism and aldosterone secretion in benign and malignant hypertension *Ann int Med* 1960 b 53 259-272 (113)
- Lassen N A O Munck and J H Thaysen Oxygen consumption and sodium reabsorption in the kidney *Acta physiol scand* 1961 51 371-384 (45)
- Lassiter W E C W Gottschalk and M Mylle Micropuncture study of net trans tubular movement of water and urea in non-diuretic mammalian kidney *Amer J Physiol* 1961 200 1139-1147 (18 21 22 39)
- Lassiter W E M Mylle and C W Gottschalk Net transtubular movements of water and urea in saline diuresis *Amer J Physiol* 1964 206 669-673 (55)
- Latta H and A M Maunsbach The juxtaglomerular apparatus as studied electron microscopically *J Ultrastruct Res* 1962 ■ 547-561 (98)
- Lever A F and W S Peart Pressor material in renal lymph *J Physiol (Lond)* 1961 159 P 35-36 (94)
- Lever A F and M Tree The estimation of renin in rabbit plasma *J Physiol (Lond)* 1963 166 P 26-27 (91)
- Leysac P P Dependence of glomerular filtration rate on proximal tubular reabsorption of salt *Acta physiol scand* 1963 58 236-242 (62 63 64)
- Leysac P P The in vivo effect of angiotensin on the proximal tubular reabsorption of salt in rat kidneys *Acta physiol scand* 1964 a 62 436-448 (53 65 67, 82 92)
- Leysac P P The effect of partial clamping of the renal artery on pressures in the proximal and distal tubules and peritubular capillaries of the rat kidney *Acta physiol scand* 1964 b 62 449-456 (89 104 106 108 110)



- Leyssac P P Some characteristics of the proximal tubular wall related to reabsorption during luminal occlusion following interruption of glomerular filtration *Acta physiol scand* 1965 a 63 36-45 (34 44 62 63 74 87)
- Leyssac P P The in vivo effect of angiotensin and noradrenaline on the proximal tubular reabsorption of salt in mammalian kidneys *Acta physiol scand* 1965 b 64 167-175 (63 75 79 80 81 82 115)
- Leyssac P P The luminal occlusion time of proximal tubules in kidneys of young rats *Acta physiol scand* 1965 c 64 176-181 (63 64)
- Lichtfield J H and P A Bott Micropuncture study of renal excretion of water, Na and Cl in the rat *Amer J Physiol* 1962 203 667-670 (40)
- Little J R and J R Robinson Composition and functional distension of the rat renal cortex *J Physiol (Lond)* 1963 165 542-549 (62)
- Lowe R D Factors controlling the release of renin from the kidney *Lancet* 1964 II 183-185 (99)
- MacRobbie E A C and H H Usung Osmotic behaviour of the epithelial cells of frog skin *Acta physiol scand* 1961 53 348-365 (34 78)
- Malm G R M Klose and G Giebisch Micropuncture study of renal potassium excretion in the rat *Amer J Physiol* 1964 206 674-686 (40)
- Marsh D K J Ullrich and G Rummich Micropuncture analysis of the behaviour of potassium ions in rat renal cortical tubules *Pflügers Arch ges Physiol* 1963 277 107-119 (35 37 40)
- Marshall E K jr A comparison of the function of the glomerular and aglomerular kidney *Amer J Physiol* 1930 94 1-10 (14)
- Maunsbach A B S C Madden and H Latta Variations in fine structure of renal tubular epithelium under different conditions of fixation *J Ultrastruct Res* 1962 6 511-530 (45)
- McManus J F A Further observations on the glomerular root of the vertebrate kidney *Quart J Microscopical Sci* 1947 88 39-43 (98)
- Mueller C B A Surtshin M R Carlin and H L White Glomerular and tubular influences on sodium and water excretion *Amer J Physiol* 1951 165 411-422 (21 58)
- Mulrow P J and W F Ganong Stimulation of aldosterone secretion by angiotensin II A preliminary report *Yale J Biol Med* 1961 33 386-395 (113)
- Oberling Ch and P Y Hatt Etude de l'appareil juxtaglomerulaire du Rat au microscope électronique *Ann d Anat Path* 1960 5 441-474 (98)
- Oken D I G Whittembury E E Windhager H J Schatzmann and A K Solomon Active sodium transport by the proximal tubule of the Necturus kidney *J clin Invest* 1959 38 1079 (36)
- Oliver J and M MacDowell Studies by microdissection and micropuncture of the abnormal kidney. The structural aspect *Proc II int Congr Nephrol Prague 1963 Excerpta Med Found Amsterdam* 1964 13-20 (17)
- Pappenheimer J R Über die Permeabilität der Glomerulusmembranen in der Niere *Klin Wschr* 1955 33 362-365 (69)
- Pickering G W M Prinzmetal and A R Kelsall The assay of renin in rabbits with experimental renal hypertension *Clin Sci* 1939-42 4 401-420 (93)
- Pitts R F *Physiology of the Kidney and Body Fluids* Year Book Med Publ Inc Chicago 1963 pp 243 (55 112)
- Rector F C jr H A Bloomer and D W Seldin Effect of potassium deficiency on the reabsorption of bicarbonate in the proximal tubule of the rat kidney *J clin Invest* 1964 43 1976-1982 (41)

- Rehberg P B Studies on kidney function I The rate of filtration ~~and reabsorption~~ in the human kidney *Biochem J* 1926 a 20 447-460 (13 12)
- Rehberg P B Studies on kidney function II The excretion of ~~urea and creatinine~~ analysed according to a modified filtration reabsorption theory *Exp Med Biol* 1926 20 461-482 (14 18 39)
- Rehberg P B Ueber die Bestimmung der Menge des Glomerulums ~~und die~~ nun als Nierenfunktionsprüfung nebst einigen Bemerkungen über ~~die~~ Harnbereitung *Zbl inn Med* 1929 50 367-377 (16 18)
- Rhodin J *Anatomy of Kidney Tubules* In *Int Rev Cytology* Eds ~~Davidson and~~ J F Danielli Acad Press Inc New York 1958 III 423-532 (5)
- Roemmelt J C O W Sartorius and R F Pitts Excretion and reabsorption ~~of water and~~ and water in the adrenalectomized dog *Amer J Physiol* 1949 159 247-254
- Rosenberg Th On accumulation and active transport in biological systems - dynamic considerations *Acta chem scand* 1948 2 14-33 (28)
- Schmid H E jr Renin a physiologic regulator of renal hemodynamics *Am J Physiol* 1962 11 185-190 (99)
- Schmidt Nielsen B Urea excretion in white rats and kangaroos ~~and~~ excitement and by diet *Amer J Physiol* 1955 181 131-139
- Schmidt Nielsen B and R O Dell Structure and concentration in the mammalian kidney *Amer J Physiol* 1961 200 1119-1124
- Schmidt Nielsen B *Desert Animals: Physiological problems of life in the desert* Univ Press London 1964 pp 277 (118)
- Scornik O A and A C Paladini Significance of blood angiotensin ~~in~~ experimental conditions *Canad Med Ass J* 1964 90 962-963
- Selkurt E E Effect of pulse pressure and mean arterial pressure on renal hemodynamics and electrolyte and water excretion. *Circ Res* 1956 (21 38)
- Selkurt E E, *Kidney Ann Rev Physiol* 1951 b 13 233-
- Selkurt E E Sodium excretion by the mammalian kidney *Am J Physiol* 1951 287-333 (57)
- Selkurt E E Ph. W Hall and M P Spencer Influence of ~~renal~~ decrement on renal clearance of creatinine *P Am J Physiol* 1949 159 369-378 (21)
- Selkurt E E and R S Post Renal clearance of sodium in the dog *Am J Physiol* 1949 159 369-378 (21)
- Sellwood R V and E B Verney The effect of water and ~~urea~~ on the renal plasma and glomerular filtrate flows *Phil Trans R Soc Lond B* 1954-55 238 361-396 (15)
- Shannon J A The renal excretion of creatinine in the dog *Am J Physiol* 1938 127 403-410 (13)
- Shannon J A Excretion of phenol red by the dog *Am J Physiol* 1938 127 602 (15)
- Shannon J A and S Fisher The renal tubular reabsorption of ~~urea~~ dog *Amer J Physiol* 1938 127 765-774 (15)
- Shannon J A and H W Smith The excretion of ~~urea~~ and phlorizinized man *J clin Invest* 1935 14 3
- Sharp G W G and A Leaf Studies on the biology of ~~the~~ *J clin Invest* 1963 42 978 (34)
- Shipley R E and R E Study Changes in renal glomerular filtration rate tissue pressure and renal artery blood pressure *Amer J Physiol* 1961 191

- Shipp J C I B Hanenson F E Windhager H J Schatzmann G Whuttembury H Yoshimura and A K Solomon Single proximal tubules of the Necturus kidney Methods for micropuncture and micropfusion *Amer J Physiol* 1958 195 563-569 (19)
- Skeggs L T J R Kahn and N P Shumway, The preparation and function of the hypertensin converting enzyme *J exper Med* 1956 103 (295-299 (77)
- Skeggs L T J R Kahn K Lentz and N P Shumway The preparation purification and amino acid sequence of a polypeptide renin substrate *J exper Med* 1957 106 439-453 (77)
- Skinner S L J W McCubbin and I H Page Renal baroreceptor control of renin secretion *Science* 1963 141 814-816 (95 97)
- Skou J C The influence of some cations on the adenosine triphosphatase from peripheral nerves *Biochim Biophys Acta* 1957 23 391-401 (46)
- Skou J C Preparation from mammalian brain and kidney of the enzyme system involved in active transport of  $\text{Na}^+$  and  $\text{K}^+$  *Biochim Biophys Acta* 1962 58 314-325 (46)
- Skou J C *Enzymatic aspects of active linked transport of  $\text{Na}^+$  and  $\text{K}^+$  through the cell membrane* In Progress in Biophysics Pergamon Press Oxford 1964 14 131-166 (46)
- Smith H W *The Kidney* Oxford Univ Press New York 1951 pp 1049 (17 18 19 21 22 26 52)
- Solomon S Transtubular potential differences of rat kidney *J cell comp Physiol* 1957 49 351-365 (20)
- Sonnenberg H and P Deetjen Methode zur Durchstromung einzelner Nephronabschitte *Pflugers Arch ges Physiol* 1964 278 669-674 (61)
- Sperber I Studies on the mammalian kidney *Zool Bidrag fran Uppsala* 1944 22 249-431 (119)
- Stein J D jr L L Bennett A A Batts and H C Li Sodium potassium and chloride retention produced by growth hormone in the absence of the adrenals *Amer J Physiol* 1952 171 587-591 (112)
- Steinhausen M I Irawanu G E Schubert and R Taugner Auflichtmikroskopie und Histologie der Tubulusdimensionen bei verschiedene Diureseszustanden *Nierkrankh Arch path Anat* 1963 376 503-527 (63 65)
- Swann H G The functional distention of the kidney A review *Tex Rep Biol Med* 1960 18 566-595 (62)
- Swann H G A new theory concerning the primary stimulus in renal hypertension *Amer J Cardiol* 1961 8 899-905 (103)
- Thurau K Autoregulation of renal blood flow and glomerular filtration rate including data on tubular and peritubular capillary pressures and vessel wall tension *Circ Res* 1964a 15 Suppl 1 132 146 (2a)
- Thurau K Fundamentals of renal circulation *Proc II int Congr Nephrol Prague 1963 Excerpta Med Found Amsterdam* 1964b 51-61 (26 104)
- Thurau K. Renal hemodynamic. *Amer J Med* 1964c 36 698-719 (112)
- Thurau K and P Deetjen Die Diuresis bei arteriellen Drucksteigerungen *Pflugers Arch ges Physiol* 1962 274 567-580 (107)
- Thurau K and E Wober Zur Localisation der autoregulativen Widerstandsänderungen in der Niere (Mikropunktionstudie) *Pflugers Arch ges Physiol* 1962 274 553-566 (25 108)
- Tobian L Interrelationship of electrolytes juxtaglomerular cells and hypertension *Physiol Rev* 1960 40 280-312 (77 93 99)
- Tobian L J Janacek and A Tomboulou Correlation between granulation of juxta glomerular cells and extractable renin in rats with experimental hypertension *Proc Soc exper Biol N Y* 1959 100 94-100 (93)

- Ullrich K J G Rummich and G Fuchs Wasserpermeabilität und transtubuläre Wasserfluss corticaler Nephronabschnitte bei verschiedenen Diuresezuständen *Pflugers Arch ges Physiol* 1964 280 99-119 (42)
- Ullrich K J, H Schmidt Nielsen R O Dell G Pehling C W Gottschalk W E Lasiter and M Mylle Micropuncture study of composition of proximal and distal tubular fluid in rat kidney *Amer J Physiol* 1963 204 527-531 (29, 39)
- Ussing H H The active ion transport through the isolated frog skin in the light of tracer studies *Acta physiol scand* 1949 a 17 1-37 (33)
- Ussing H H The distinction by means of tracers between active transport and diffusion The transfer of iodide across the isolated frog skin *Acta physiol scand* 1949 b 19 43-56 (29 30)
- Ussing H H Transport of ions across the cellular membrane *Physiol Rev* 1949 29 127-145 (28)
- Ussing H H The alkali metal ions in isolated systems and tissues In Ussing H H P Kruhoffer J H Thaysen and N A Thorn The Alkali Metal Ions in Biology Springer Verlag Heidelberg 1960 pp 598 1-195 (32 37)
- Ussing H H and K Zerahn Active transport of sodium as the source of electric current in the short circuited isolated frog skin *Acta physiol scand* 1951 23 110-127 (79)
- Walker A M B A Bott J Oliver and M MacDowell The collection and analysis of fluid from single nephrons of the mammalian kidney *Amer J Physiol* 1941 134 580-595 (18 22 54 55)
- Wallenius G Renal clearance of dextran as measure of glomerular permeability Almquist and Wiksells Uppsala 1954 pp 91 Thesis (70)
- Vander A J, R L Malvin W S Wilde J Lapidus L P Sullivan and V M McMurray Effects of adrenalectomy on proximal and distal tubular sodium reabsorption *Proc Soc exp Biol N Y* 1958 99 323-325 (88)
- Vander A J and R Miller Control of renin secretion in the anesthetized dog *Amer J Physiol* 1964 207 537-546 (95 101 103)
- Vander A J W S Wilde and R L Malvin Stop flow analysis of aldosterone and steroidal antagonist SC 8109 on renal tubular sodium transport kinetics *Proc Soc exp Biol N Y* 1960 103 525-527 (88)
- Waugh W H and R H Shanks Cause of genuine autoregulation of the renal circulation *Circ Res* 1960 8 871-888 (104 107)
- Wearn J T and A N Richards Observations on the composition of glomerular urine with particular reference to the problem of reabsorption in the renal tubules *Amer J Physiol* 1924 71 209-227 (13)
- Wesson L G jr Glomerular and tubular factors in the renal excretion of sodium chloride *Medicine* 1957 36 281-396 (19 60)
- Wesson L G jr W P Anslov jr L G Raisz A A Bolomey and M Ladd Effect of sustained expansion of extracellular fluid volume upon filtration rate renal plasma flow and electrolyte and water excretion in the dog *Amer J Physiol* 1950 167 677-686 (21)
- Wesson L G jr W P Anslov jr and H W Smith The excretion of strong electrolytes *Bull N Y Acad Med* 1948 24 586-606 (19 20 21 88)
- Veyrat R J de Champlain R Boucher and J Genest Measurement of human renin activity in some physiological and pathological states *Canad Med Ass J* 1964 90 215-220 (96)
- Whittembury G Ion and water transport in the proximal tubules of the kidney of *Necturus maculosus* *J gen Physiol* 1960 43 II 43-56 (20 36)
- Whittembury G Sodium for potassium exchange in kidney slices *Abstr int Biophys Meeting Paris* 1964 (35 44)

- Whittembury C Sodium extrusion and potassium uptake in Guinea pig kidney cortex slices *J gen Physiol* 1965 48 699-717 (35 44)
- Wilbrandt W and Th Rosenberg The concept of carrier transport and its corollaries in pharmacology *Pharmacol Rev* 1961 13 109-183 (30 31)
- Windhager E E and G Giebisch Comparison of short circuit current and net water movement in single perfused proximal tubules of rat kidney *Nature* 1961 a 191 1205-1207 (20 30 36 64)
- Windhager E E and G Giebisch Micropuncture study of renal tubular transfer of sodium chloride in the rat *Amer J Physiol* 1961 b 200 581-590 (18 20 22 30 40 55)
- Windhager E E G Whittembury D E Oken H J Schatzmann and A H Solomon Single proximal tubules of the Necturus kidney III Dependence of H<sub>2</sub>O movement on sodium chloride concentration *Amer J Physiol* 1959 197 313-318 (20 35)
- Wirz H Die Druckverhältnisse in der normalen Niere *Schweiz med Wochenschr* 1956 86 377-394 (108)

## Subject index

Numbers refer to pages of the present review in which the subject is discussed  
 letters (A-H) refer to previously published papers in which the subject is discussed  
 Previously published papers are enlisted in front of this review

- Active transport*  
 definitions 14 20 28  
 of chloride 39  
 of potassium 38  
 of sodium 19-20 37-38 G H
- Adenosin triphosphatase (ATP ase)*  
 46 112 G
- Aldosterone*  
 effect on active sodium transport 34  
 effect on distal sodium reabsorption 88  
 114  
 in plasma 114  
 regulation of secretion 113-115 116 B
- Angiotensin* 77  
 in plasma 95-97  
 on aldosterone secretion 113-114 E  
 on ATP ase 112  
 on oxygen consumption 112  
 proximal tubular effect of (on Tm)  
 77-85 106 109 110 B D E  
 vascular effect of 77 104 110 C D
- Angiotensinase* 92 101
- Angiotensinogen* 77 94 101 107
- Autoregulation* 25-26 104 105 106-108
- Carrier* 30-31 38 47-48
- Colloid osmotic pressure* 24 59 67 91  
 113
- Concentrating capacity* 118-119
- Effective filtration pressure* 23-26 56 59  
 69 73 89-91 B D
- Efficiency of oxygen* (in sodium transport) 45 G
- Epitheloid cells* (see also JGA) 76 77  
 104 105 D
- Filtration fraction* 90
- Filtration rate* glomerular  
 determination of 13 19 24  
 influence by pressor agents on 115-116  
 B E  
 influence by tubular reabsorption on 16  
 54 59 71-73 75 84-85 103 112  
 A-F  
 in saline diuresis 21 24 49-50 59-60  
 100  
 in water diuresis 108-109  
 in volume expansion 100-101 113  
 range of variation in 16 75 84-85  
 101 A-F  
 regulation of (see also autoregulation)  
 24 84 86 100-101 104 109-110  
 A-F
- Flux ratio* 28-29 30
- Glomerulo tubular balance* 17-18 53 58  
 104 B
- Gradient limitation* 20-21 92 29 47 55  
 103
- Intertubular space* 69 C D G H
- Juxtaglomerular apparatus*  
 evolution 117-119  
 function 75-77 87 97-101 103-110  
 117 119 G  
 morphology 76 107 G
- Juxtaglomerular Index (JGI)* 93
- Macula densa* 76 98 G
- Microvilli* 45 54 G
- Noradrenaline*  
 on angiotensin release 95 115-116  
 on plasma flow renal 115-116 E  
 on proximal reabsorption rate 79 115-  
 116 E  
 on sodium excretion 83 115-116 E

*Occlusion time* tubular 63-66 76 A  
 angiotensin effect on 79-83, B E  
 in clamped kidneys 67 85 II  
 in small rats 64-65, C  
 method evaluation of 63-64 65-67 A  
 noradrenaline effect on 79 E

*Oncotic pressure* see colloid osmotic pressure

### *Pinocytosis*

in amoebae 48 111  
 micro- 45-50 111 G

### *Plasma flow renal*

influence by pressor agents on 115-116  
 II  
 in saline diuresis 21 24 59 100 113  
 regulation of (see also autoregulation),  
 84 86 100-101 101-106 109-110  
 B C

### *Potential difference electrical*

across amphibian epithelia 32-33  
 across luminal membrane 20 36 37  
 across peritubular membrane 20 35  
 37 42 G  
 across tubular wall 20 35-37

### *Pressure*

hydrostatic (- drop across glom  
 membrane see effective filtration pres  
 sure)  
 glomerular capillary 69 89 90 104  
 117 D  
 intratubular distal 23 100 102 108  
 110 C  
 intratubular proximal (and Bowman's  
 capsular) 23-26 69 71 73-74  
 82-83 88 107 113 C D  
 - regulation of 61 87-91 104  
 106 107-108 109-110 117 119  
 C  
 peritubular capillary 25-26 73-74  
 89 103 C D  
 resistance of tubular wall to 73-74  
 D

### *glomerular propulsion*

Def 23  
 conditions of decrease in 97  
 consequences of changes in 23-24  
 26 58-59 69 71-74 90-91 100  
 105 106 109 116

### *Penin*

location 76-77 B  
 content in kidneys 92-94 99 104 117  
 in lymph 94  
 in plasma 91 95-97 101-102

*Salt* see Sodium chloride

*Saturation kinetics* (see also Tm) 30-31  
 33 48

### *Sodium chloride*

excretion fraction of 14 16  
 19 55

excretion rate of  
 at alterations in renal arterial pres  
 sure 21 58-59 107

limitations in 71-73 II

in saline loadings -isotonic 21 58-  
 59 113 114

in saline loadings -hypertonic 57-58  
 88

in osmotic diuresis 19

regulation of 87-88 116-119 B C

balance 87 96 114 C

concentration

in tubular fluid 20 22 30 37 104

intracellular 32 33 34 37 43-47  
 G H

on reabsorption rate 49-50

net transtubular flux of (see  
 also Active transport of and tubular  
 reabsorption of) 20 36 53-54 64  
 66 B H

### *Tm limitation*

definition 15 31 48 68 A F

for glucose 15 50 67 86 B

for salt 18

in amphibian epithelia 33

in distal tubules 21 68 87-88 112

in proximal tubules 50 54 56 59-  
 61 64 68 87 89 104 106 111

A-C E F

- regulation of 60 77-86 109-110

B C F

for volume 48-49 56 67 111

### *Transport transepithelial*

mechanisms of 32-33 45-51 D G H

### *Tubular*

diameters 53 63-64 65 67 110

A-C E G H

fluid volume 62-65 A II E G

occlusion 54 61-62 65-66 67

85-86 89 110 A-I G

reabsorptive capacity (see

Tm for)

reabsorption

of bicarbonate 41 42 67

of chloride 40 42 67

of glucose 15 50-51

of salt and water 17-19 42  
- at alterations in renal arterial  
  pressure 58-59 107 A-F  
- in saline loadings isotonic 21  
  55 59-60  
  hypertonic 21-22 49 55 56-58  
- in osmotic diuresis 19-21  
- in water diuresis 22 41 109  
of potassium 40 42 67  
of urea 14 22-23 39 41-42 67  
Secretion 14-15  
  of phenol red 14

*Turnover rate of filtrate (or of salt)* 71  
73 85 87 89 101 102 108 109 112

*Vasopressin*

  on intratubular pressure 100 108  
  on sodium transport 33-34 78-79 109  
  on water permeability 78

*Water permeability* proximal tubular  
42-43

*Water net flux* 43 52-53









ACTA PHYSIOLOGICA SCANDINAVICA

VOL 70 SUPPLEMENTUM 292

CIRCULATORY AND RESPIRATORY  
ADAPTATION DURING  
PROLONGED EXERCISE

BY

LARS-GORAN EKELOUND

STOCKHOLM 1967



# CONTENTS

Preface	3
Acknowledgements	6
Introduction	7
CHAPTER I	Material and Methods
	Material
	Methods and Procedure
	Comments
CHAPTER II	Blood volume changes in connection with exercise
	Previous investigations
	Present investigation
	Discussion
CHAPTER III	Adaptation of the central circulation during prolonged exercise
	Previous investigations
	Present investigation
	Discussion
	Mechanical systole
	Heart volume
	Comments
	General discussion
CHAPTER IV	Adaptation of the respiration during prolonged exercise
	Previous investigations
	Present investigation
	Discussion
CHAPTER V	General summary and conclusions



## Preface

The present publication is based mainly on studies reported in the following papers

- I Ekelund L G and A Holmgren Circulatory and respiratory adaptation during long term non steady state exercise, in the sitting position Acta physiol scand 1964 62 240—255
- II Ekelund L G and A Holmgren Steady state diffusing capacity during prolonged, non steady state sitting exercise in ordinarily trained young men Acta physiol scand 1965 65 143—152
- III Ekelund L G Circulatory and respiratory adaptation during prolonged exercise in the supine position Acta physiol scand 1966 68 382—396
- IV Ekelund L G Circulatory and respiratory adaptation during prolonged exercise of moderate intensity in the sitting position Acta physiol scand 1967 In print
- V Ekelund L G A Holmgren and C O Ovenfors Heart volume during prolonged exercise in the supine and sitting position Acta physiol scand 1967 In print

In the text these will be quoted as (I) and (II) etc





## Introduction

The adaptation of the circulation and respiration during short periods of exercise of varying intensity has been extensively studied. Recent reviews on this subject have been published by Asmussen & Nielsen (1955), Rushmer (1959), Wade & Bishop (1962) and Holmgren & Ekelund (1967). The studies have usually covered exercise periods of about 5–10 minutes which have been long enough to allow the organism to reach a relative steady state in respect of the different variables studied. Steady state in a biological system is, however, always a relative steady state, i.e. the rate of change of some variable is within certain limits. In short work tests a relative steady state in respect of heart rate is often defined as a change up to 10 beats/min between 2 and 6 min (Sjostrand 1960). Studies during prolonged continuous work for half an hour to several hours have been made by Krogh & Lindhard (1920) and Christensen & Hansen (1939). These were mainly metabolic studies with investigations of the respiratory quotients and blood glucose concentration during prolonged exercise and also of the thermo regulation. Ekelund & Holmgren (1962) gave in a preliminary report data from heart catheterization studies in six healthy men during prolonged sitting exercise. Cobb & Johnson (1963) measured the cardiac output during prolonged exercise by the dye-dilution method and also studied the free fatty acid metabolism. In 1964 Saltin & Stenberg published a report on prolonged severe exercise which was performed both on a bicycle and on a treadmill with short intermittent rest periods. Dye dilution cardiac output and the brachial artery pressure were measured in four well-trained males. From these earlier reports we know that there is often a non-steady state in respect of heart rate which is due to a fall in stroke volume.

The principal aim of the present investigations was to study the variables of the central circulation such as cardiac output, stroke volume, heart rate and the pressures in the pulmonary and systemic circulation during prolonged exercise in a defined non steady state condition. A simultaneous study of different respiratory variables was also of interest because there is often a non-steady state in respect of respiratory rate during prolonged exercise. The same type of study was performed both in the sitting (I) and supine (III) position to investigate the influence of body position, i.e. the influence of gravitation on the distribution of blood volume within the capacity vessels. To investigate the importance of the relative intensity of exercise, two studies were performed in the sitting position with different relative work loads (I–IV). For a better

## Acknowledgements

The present investigation was performed at the Department of Clinical Physiology Karolinska Sjukhuset, Stockholm

To Professor Torgny Sjostrand head of the Department, I wish to convey my warm thanks for his continuous encouragement and never failing support, and for all discussions throughout the whole investigation

Assistant Professor Alf Holmgren head of the Clinical Physiological Laboratory of the Thoracic Clinic, initiated the investigation and also took an active part in several studies and gave valuable and constructive criticism

To Assistant Professor Arne Granath head of the Clinical Physiological Laboratory of Danderyd Hospital I am most grateful for his competent and persevering help in two of the catheterization studies and for his stimulating criticism

I am deeply obliged to Carl Olof Ovenfors, Assistant Professor of the Department of Roentgenology at the Thoracic Clinics for the friendly and consistent support he gave me

I am indebted to Miss Guri Andersson for her skilful assistance in the catheterization examinations and to Miss Valborg Hagsand for drawing the diagrams and carrying out the main part of the calculations

Mrs Gertrud Grunfarb has given me excellent secretarial help

To all colleagues who have generously helped me I wish to express my sincere gratitude I am also greatly indebted to the entire staff of the laboratory for their collaboration in the collection analysis and presentation of the data

The text has been corrected by Mr John Hogg

Finally I wish to thank my wife for the understanding she has always shown and for all the labour she has devoted to this work

The study was supported by grants from Svenska Idrottens Vetenskapliga Forskningsrad, Riksidrottsforbundets poliklinikkommitté, the Swedish National Association against Heart and Chest Diseases and from Karolinska Institutet (Reservationsanslaget)

## Material and Methods

## Material

The observations in the present report were obtained from studies in 29 healthy male volunteers of ordinary physical fitness. All were blood donors registered at and controlled by the hospital blood bank. The subjects were mainly students which explains the rather narrow age range (19–30 years). Three of the subjects took part in two investigations. The anthropometric data are summarized in Table I and detailed information is given in each separate study.

*Comments.* The subjects were somewhat taller than the average for their age (mean 23  $\pm$  years) but in other respects comparable with, for example, military conscripts (Lornvall 1963). The low value for hemoglobin concentration is mainly due to the fact that the samples were taken when the subjects had rested supine for about 15 min, which gives a value about 6 per cent lower than in sitting position (Ekelund & Kajser 1966, unpublished observation) due to an increase in plasma volume.

Table I Summarized anthropometric data from studies I–V. The abbreviations are the same as in the text.

	Age years	Height cm	Weight kg	Heart vol ml	THB g	THB g/kg b w	Hb conc. g/100 ml	Blood vol l	Blood vol ml/kg b w	$W_{170}$ kpm/min sitting
$\bar{x}$	23.2	181.4	71.4	784	759	10.59	13.54	6.23	87.60	1102
$n$	29	29	29	27	27	27	27	27	27	29
SD	2.5	6.8	6.7	106	97	0.87	0.75	0.83	6.37	165
range	19–30	167–197	58–86	645–985	560–985	8.2–12.2	11.1–14.7	4.9–8.6	74.3–100.0	750–1500

## Methods and Procedure

*Methods*

Detailed descriptions of methods and procedures have been reported in the separate papers and are therefore only briefly summarized below.

*Electrocardiograms* were recorded with a direct writing ink jet recorder (Mingograf 42) both at rest in the supine and standing positions, during exercise on a bicycle ergometer in sitting position, and in the supine position after exercise, using CR leads with modifications described by Holmgren & Strandell (1961).

*Work capacity* ( $W_{170}$ ) was determined in the sitting and in 11 subjects also in the supine position by the method of Sjostrand (1960) on an electrically braked bicycle ergometer (Holmgren & Mattsson 1954) with the work load increasing stepwise every sixth minute.  $W_{170}$  is defined as the rate of work in kpm/min which a subject could perform at a heart rate of 170 beats/min. The value of  $W_{170}$  was obtained by inter- or extrapolation using the approximately linear relationship between pulse rate and work load.

analysis of the findings from the heart catheterization, a study of the total heart volume determined by X ray was performed during prolonged exercise both in sitting and supine position (V) To further evaluate the respiratory adaptation during prolonged exercise another study was performed which incorporated measurements of steady state diffusing capacity during prolonged exercise in the sitting position (II)

and base excess were determined according to Andersen et al (1960). Arterial oxygen tension ( $P_{O_2}$ ) was measured with a Clark microcathode electrode (polypropylene membrane) in a stainless steel cuvette kept at 37°C. Measurements of pH,  $P_{CO_2}$  and  $P_{O_2}$  were corrected for differences between body temperature and electrode temperature (Holmgren & McIlroy 1964).

The mechanical efficiency was calculated from the equation

$$M = \frac{(\text{rate of work lpm/min}) \cdot 100}{(\text{oxygen uptake l/min exercise} - \text{oxygen uptake l/min rest}) \cdot 4.9 \cdot 427}$$

Lactic acid concentration was determined in arterial blood by the colorimetric method of Barker & Summerson (1941) as modified by Strom (1949).

**Mechanical systole** The mechanical systole was measured from recordings of the brachial artery pressure with a paper speed of 100 mm/sec. Mean values were calculated from at least 3 usually 4–5 consecutive heart cycles. The tip of the brachial artery catheter was usually introduced 3 dm in the central direction.

**Body temperature** The body temperature was measured as rectal temperature with a thermocouple (Ellab Copenhagen Type TE 3). The rectal applicator was inserted to a depth of about 5 cm from the sphincter ani.

**Statistical calculations** were mainly performed according to Dahlberg (1940) and Snedecor (1959). Differences between regression lines were tested according to Hald (1960). The following probability ( $P$ ) levels of significance were used:  $P < 0.001$  highly significant,  $0.001 < P < 0.01$  significant, and  $0.01 < P < 0.05$  probably significant.

### Procedure

The study started with determinations of some measures of the circulatory system such as total amount of hemoglobin, total blood volume, heart volume and rate of work at pulse rate 170 ( $W_{170}$ ). A pilot test was also performed to select a work load which the subjects could sustain for about one hour in the decided body position. The first four studies (I–IV) were performed in the morning after the subjects had had a light meal about one hour before the experimental procedure. No sedative or quinine was given. The room temperature was between 20 and 24.5°C with a relative humidity of about 40 per cent. The fluid losses (blood and sweat) were compensated for by continuous saline administration to keep the body weight constant (weight loss  $< 0.7$  kg). Small amounts of water were also given per os. Study V was performed later in the day about four hours after lunch. In this study no compensation was made for fluid losses. No complications occurred during or after any of the experiments.

### Comments

All methods used during the different investigations were regularly checked with regard to validity and reproducibility. The reproducibility calculated from duplicate estimations for every method agrees very well with the figures published for each separate method from this and other laboratories. Table II shows the errors in  $W_{170}$ , heart volume (prone), cardiac output and stroke volume, lactic acid, pH,  $P_{CO_2}$ ,  $P_{O_2}$  and diffusion capacity of the lungs taken from Hellstrom & Holmgren (1966), Holmgren & Pernow (1960), Holmgren (1965b) and Strandell (1964b). The remaining values were calculated from measurements on the actual subjects plus ordinary subjects who took part in metabolic studies during the same period.

All measurements with the exception of  $W_{170}$ , heart volume (prone), oxygen uptake, cardiac output, stroke volume and diffusion capacity were made in duplicate.

Table II Errors for a single determination as estimated from duplicate determinations

Variable	Experimental conditions	No of duplicate determinations	Mean value	Error $\pm \sqrt{\frac{\sum d^2}{2n}}$	Error in per cent of the mean value
$W_{170}$ kpm/min	Sitting	56	1135	55.4	4.9
Tot Hb g	CO method semirecumbent	50	735	27.4	3.7
Blood vol l	CO method semirecumbent	50	5.43	0.239	4.4
Blood vol l	$I^{125}$ albumin supine	49	5.04	0.224	4.4
Hemoglob conc g/100 ml	supine	40	13.77	0.095	0.69
Heart vol ml	prone	112	785	33.4	4.3
Heart vol ml	sitting exercise	44	777	38.1	4.9
Heart vol ml	supine exercise	34	888	36.7	4.1
$V_O$ ml STPD/min	supine rest	35	289	10.6	3.7
$V_O$ ml STPD/min	supine exercise	35	1829	42	2.3
Oxygen saturat	exercise art	104	96.9	0.35	—
	ven	103	41.8	0.34	—
Card output l/min	supine exercise	27	11.1	0.58	5.2
Stroke vol ml	supine exercise	26	92	6.3	6.8
Lact acid meq/l	arter blood	60	1.28	0.122	9.5
Lact acid meq/l	»	60	2.75	0.220	8.0
Lact acid meq/l	»	60	5.00	0.160	3.2
RQ	rest	35	0.773	0.031	3.9
RQ	exercise	35	0.818	0.025	3.1
$P_{CO}$ mm Hg	Severinghaus	85	40.0	0.198	0.5
$P_{CO}$ mm Hg	Astrup	39	37.0	0.93	2.5
pH units	Microelectr Astrup	50	7.383	$2.2 \cdot 10^{-3}$	0.03
$P_O$ mm Hg	Clark electr	100	77.5	0.93	1.2
$DL_{CO}$ ml/min/mm Hg	sitting exercise	13	37.9	2.7	7.2
Mech syst c sec	Sitting rest + exer	50	23.7	0.43	1.8

## CHAPTER II

# Blood volume changes in connection with exercise

### Previous investigations

Several earlier investigations (e.g. Thompson Thompson & Dailey 1928) have shown that plasma volume decreases on change of posture from supine to sitting and shortly after starting exercise both in supine and sitting position. Before isotope methods became available two explanations had been proposed to account for this change: the displacement of red cells from blood depots into the circulation or a concentration of the blood due to passage of fluid from the blood into the tissues. Nylin (1947) found no change of the circulating red cell volume studied by isotope techniques in connection with exercise. Uehlinger & Buhlmann (1961) studied the red cell volume during exercise by an isotope technique and at the same time the plasma volume with  $I^{131}$  albumin. They injected chromium labelled erythrocytes; however, and labelled albumin only once before exercise and then measured the changes of concentration after estimation of the change of slope for  $I^{131}$  albumin. They found an unchanged erythrocyte volume and a decrease in plasma volume by, on an average, 10 per cent in sitting position and 3.6 per cent in supine position. The exercise was of moderate absolute intensity (corresponding to an oxygen uptake 1–1.5 l/min) during 5 minutes. Fricke (1965) studied the changes in red cell volume and plasma volume during exercise with isotope labelled erythrocytes and several injections of Evans blue. The exercise was performed in the supine position and introduced no change in the red cell volume, but there were significant changes in plasma volume, with 5 to 20 per cent decrease from rest to exercise. There was a correlation between the plasma volume decrease in per cent and the relative work load (expressed as per cent of  $W_{170}$ ). The relation between total body hematocrit and peripheral blood hematocrit increased by, on an average, 3.2 per cent during exercise, from 0.88 at rest to 0.90 during exercise. On the assumption that changes in hemoglobin concentration reflected changes in total blood volume, Holmgren (1956) estimated the decrease in blood volume during exercise and found already after 5 min exercise a decrease which after 15 min exercise amounted to 7 per cent of the resting value. During prolonged continuous exercise of 30 min duration there was no further change in hemoglobin concentration. Saltun & Stenberg (1964)



Table II Errors for a single determination as estimated from duplicate determinations

Variable	Experimental conditions	No of duplicate determinations	Mean value	Error $\pm \sqrt{\frac{\sum d^2}{2n}}$	Error in per cent of the mean value
$W_{110}$ kpm/min	Sitting	56	1135	55.4	4.9
Tot Hb g	CO method semirecumbent	50	735	27.4	3.7
Blood vol l	CO method semirecumbent	50	5.43	0.239	4.4
Blood vol l	1st albumin supine	49	5.04	0.224	4.4
Hemoglob conc g/100 ml	supine	40	13.77	0.095	0.69
Heart vol ml	prone	112	785	33.4	4.3
Heart vol ml	sitting exercise	44	777	38.1	4.9
Heart vol ml	supine exercise	34	888	36.7	4.1
$V_O$ ml STPD/min	supine rest	35	289	10.6	3.7
$V_O$ ml STPD/min	supine exerc sitt	35	1829	42	2.3
Oxygen saturat %	exercise art	104	96.9	0.35	—
	ven	103	41.8	0.34	—
Card output l/min	supine exercise	27	11.1	0.58	5.2
Stroke vol ml	supine exercise	26	97	6.3	6.8
Lact acid meq/l	arter blood	60	1.28	0.122	9.5
Lact acid meq/l	»	60	2.75	0.220	8.0
Lact acid meq/l	»	60	5.00	0.160	3.2
RQ	rest	35	0.773	0.031	3.9
RQ	exercise	35	0.818	0.025	3.1
$P_{CO}$ mm Hg	Severinghaus	85	40.0	0.198	0.5
$P_{CO}$ mm Hg	Astrup	39	37.0	0.93	2.5
pH units	Microelectr Astrup	50	7.383	$2.2 \cdot 10^{-2}$	0.03
$P_O$ mm Hg	Clark electr	100	77.5	0.93	1.2
$DL_{CO}$ ml/min/mm Hg	sitting exercise	13	37.9	2.7	7.2
Mech syst. sec	Sitting rest — exer	50	23.7	0.43	1.8

by Fricke (1965) who estimated red cell volume with radioactive chromium and plasma volume with Evans blue. During the present investigations the  $I^{131}$  albumin was carefully checked for free iodine and denaturation of the albumin; the presence of the latter would have given too high blood volumes especially during exercise. In the first investigation samples were drawn from two of the subjects at different times after the injection to check the mixing time and that not too much of the albumin was lost from the intravascular space. The loss from the intravascular space was found to be less than 2 per cent and therefore no correction was made. The error which may be introduced if the elimination of albumin is more rapid during exercise than at rest is an underestimation of the blood volume decrease on start of exercise. There is no reason why the elimination should change during exercise and as a consequence the values during exercise should be valid.

## CHAPTER III

# Adaptation of the central circulation during prolonged exercise

### Previous investigations

Cobb & Johnson (1963) published a study of some hemodynamic variables during prolonged exercise in a sedentary group of 14 males and in a physically active group of 7 males. The subjects walked on a treadmill with the same load for both groups which gave an oxygen uptake of about 1.1 litre/min. This corresponded to a high relative work load for the sedentary group, with a heart rate after 10 min of 176 beats/min. The sedentary subjects could not walk for more than 15–36 minutes whereas the physically active group with a 10 min heart rate of 141 beats/min continued for at least one hour. In both groups the dye dilution cardiac output was on an average constant over the whole work period, with small but insignificant changes. Heart rate rose progressively and significantly during exercise in all subjects with a corresponding decrease in stroke volume. In the sedentary group the average fall was 16 per cent from 4 to 6 min until the end of exercise. In the physically active group the fall in stroke volume was 14 per cent from 4 to 6 min to the end of exercise (40–60 min). No pressures were reported. Corresponding findings have been reported by Saltin & Stenberg (1964) who studied four well trained males during 180 min work both on treadmill and bicycle ergometer with an oxygen uptake of 75 per cent of the individuals maximal uptake, which corresponded to an oxygen uptake between 2.3 and 3.4 litre/min.

In a study of 20 min treadmill exercise Levy, Tabakin & Hanson (1961) found that the cardiac output measured by dye dilution was maintained steady for 7 of the 12 subjects over the whole period and in all subjects between 8 and 20 min of exercise. The oxygen uptake and ventilation were unchanged, but no values of heart rate, stroke volume or pressures were given. In another dye dilution study (Grimby, Nilsson & Sanne 1966) 11 males exercised for 30 min in sitting position on a bicycle ergometer; there were no significant changes in cardiac output but a small decrease in stroke volume corresponding to an increase in heart rate.

### Present investigation

#### *Cardiac output*

In the 18 subjects studied with heart catheterization (I, III, IV) in different body positions and at different relative intensities, cardiac output was meas

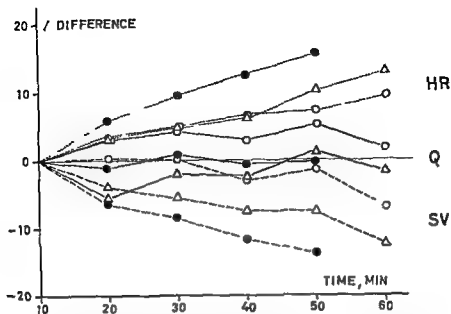


Fig 1 Response of heart rate HR (●) cardiac output Q (—) and stroke volume SV (—) during prolonged exercise mean values for six subjects in sitting study I (●) study II (○) and in supine position study III (△) The data are presented as a percentage difference from the values obtained after 10 min exercise

ured every 10th minute during continuous work on a bicycle ergometer for 50—60 minutes. There were no significant changes in cardiac output (Fig 1), but individual changes up to  $\pm 1$  litre/min, the individual changes in cardiac output being followed by opposite changes in arterio-venous oxygen difference. The more or less marked increase in oxygen uptake (see chapter IV) was followed by a corresponding increase in arterio-venous oxygen difference. The change in cardiac output was not correlated to changes in respiratory rate, total ventilation or body temperature. The 10 min cardiac outputs fell well within the normal limits of cardiac output and oxygen uptake in the different body positions which have been published from this laboratory (Bevegård, Holmgren & Jonsson 1960).

### Heart rate

Heart rate increased continuously (measured for 1 minute every 10th min) in all studies, both of circulation (I, III, IV) and of respiration and heart volume (II, V) (Fig 1). The increase in heart rate was most marked in studies I and II.

where the relative work load was about 77 per cent of  $W_{170}$  corresponding to a 10 min heart rate of 148 beats/min in study I. In studies with lower relative intensity (III, IV) there was also a significant increase in heart rate but not of as great magnitude as in the first study. The slope of heart rate with time was steeper in the first 30 min than during the last 30 min of exercise. These questions have been studied in detail by Ahlborg (1967). In the group of 18 subjects who took part in the heart catheterization studies there was a probably significant correlation between the increase in heart rate from 10 min to end of exercise and the relative work load either expressed as pulse rate after 10 min exercise or per cent of  $W_{170}$   $r = 0.56$  for both. The increase in heart rate from 10 min to the end of exercise was in the present material highly significantly correlated to the increase in body temperature during the same period  $r = 0.72$ . The heart rate during exercise was also significantly correlated to body temperature in 16 subjects with individual correlation coefficients between 0.87 and 1.00 ( $n = 5-6$ ) and  $r = 0.64$  for the whole group ( $n = 104$ ). The increase in heart rate from 10 min to the end of exercise in the group of 18 subjects was not correlated to increase in total ventilation, orthostatic pulse increase, lactic acid concentration after 10 min exercise, decrease in total blood volume from rest to 10 min exercise, or increase in heart rate at a pulse rate of about 140 beats/min between 11 and 12 min exercise in the ordinary bicycle test.

### *Stroke volume*

In all three circulatory studies (I-III-IV) there was a significant decrease in stroke volume (Fig. 3) the magnitude of which was related to the magnitude of the increase in heart rate during the prolonged exercise for which reason the fall in stroke volume was most pronounced in the first study in sitting position at the highest relative work load. Between the two studies at the same relative work load in different body positions (III and IV) there was no difference in respect of stroke volume decrease. For the group of 18 subjects there was a significant correlation between decrease in stroke volume between 10 min and end of exercise ( $\Delta SV_{10-end}$ ) and the corresponding heart rate values ( $\Delta HR_{10-end}$ ).

$$\Delta SV_{10-end} \text{ (ml)} = 1.66 + 0.663 \Delta HR_{10-end} \quad r = 0.60 \quad S.D. = 7.98$$

The decrease in stroke volume shows the same relation to the other variables as the increase in heart rate mentioned above so that the decrease in stroke volume is probably significantly related to the relative work load expressed as heart rate after 10 min work or per cent of  $W_{170}$ . The 10 min values fell within

the normal range of variation for the relationship between stroke volume during exercise and total amount of hemoglobin and heart volume (Holmgren, Jonsson & Sjostrand 1960 Bevegård et al 1960) in the different body positions

### Intravascular pressures

In the three catheterization studies (I, III, IV) the pressures in the right ventricle, pulmonary artery and brachial artery were measured at rest and every 10th min during work. The pulmonary wedge (PCV) pressure was recorded at rest in 16 subjects but at the beginning and end of exercise only in seven subjects. On transition from rest to exercise all pressures rose to values which fell within normal limits in relation to cardiac output reported earlier from this laboratory (Holmgren & Ekelund 1967). During the continuous prolonged exercise there was a more or less continuous decrease of the pressures in the right ventricle, pulmonary artery and brachial artery (Fig 2). The pressure

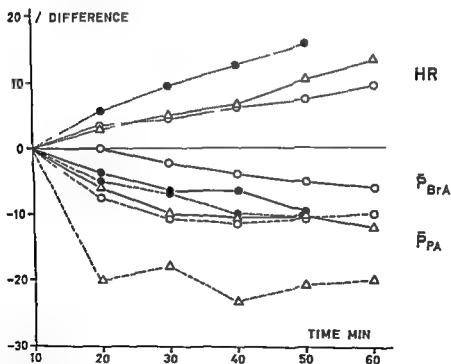


Fig 2 Response of heart rate HR ( ) brachial artery mean pressure  $\bar{P}_{BrA}$  ( — ) and pulmonary artery mean pressure  $\bar{P}_{PA}$  ( ) during prolonged exercise. For further data see fig 1

decrease in the brachial artery from 10 min to the end of exercise (50–60 min exercise) was of the order of 6–10 mm Hg with largest decrease in the group with the highest relative work load. The magnitude and pattern of decrease in the mean pulmonary artery pressure was quite different in the two body positions. In the sitting position, both at a high relative work load and at a medium work load, the decrease in pulmonary artery mean pressure was continuous and amounted maximally to 2 mm Hg at the end of exercise. In the supine position on the other hand, the decrease was pronounced already between 10 and 20 min exercise (also observed by Sancetta, 1957), after which it remained almost unchanged. The maximal decrease was on an average 5.2 mm Hg and was mainly a result of a decrease in the diastolic pressure in the pulmonary artery. The end diastolic pressure in the right ventricle decreased in all three studies by 2.2–2.6 mm Hg during the continuous exercise. There was the same magnitude and pattern of change in all three studies. The PCV pressure showed no clear cut changes but should probably decrease as much as the diastolic pressure in the pulmonary artery because this is equal to the PCV pressure in normal subjects (Holmgren & Ekelund 1967). In supine position the PCV pressure was only recorded in two subjects during exercise and decreased in both. In the sitting position at medium level of work intensity the PCV pressure was recorded in five subjects during exercise and was in these cases unchanged.

## Discussion

### *Cardiac output and metabolic requirements*

In order to exercise continuously the working muscles have to receive a constant amount of oxygen per time unit plus the necessary fuel for combustion, such as free fatty acids and carbohydrates. The two most important sources of energy are the circulating free fatty acids and local fat depots together with muscle glycogen (Havel et al. 1964 a, Ahlborg et al. 1967). The relation between these two main sources varies according to nutritional state, relative work intensity and physical fitness. The relation also changes during a work period as has been shown by RQ estimations during prolonged exercise (Christensen & Hansen 1939). In the present study the oxygen uptake remained steady or increased slightly when the work was of a high intensity. The increase in oxygen uptake at a constant work load might partly be a result of a less efficient type of exercise in which more muscle groups are used at the end of exercise than at the beginning. Another explanation could be that the combustion of fat requires somewhat more oxygen for each unit of energy compared with the combustion of carbohydrates about 10 per cent (Krogh & Landhard 1920).

At the end of exercise less of the energy is delivered from combustion of carbohydrates than at the beginning as indicated by the decreasing RQ levels and this will require more oxygen.

In the present study the cardiac output was maintained constant and the necessary increase in oxygen to the exercising muscle was supplied by an increased extraction of oxygen from the blood i.e. an increased arterio-venous oxygen difference. During the type of exercise considered any local demand for an increased flow to comply with the requirement of the heat regulation does not seem to influence the total cardiac output because there is no correlation between changes in cardiac output and body temperature. Such local demands may be met by a change of regional perfusion in the intestinal organs such as kidneys and liver. Several studies have shown that there is a marked decrease in blood flow to the kidney and to the liver on transition from rest to exercise (Grimby 1965, Castenfors 1967, Wade & Bishop 1962, Rowell et al 1965, Hultman 1966). Some of these authors report values from prolonged exercise which indicate that an additional amount of blood may be shunted from the kidney and liver at the end of prolonged heavy exercise. Castenfors (1967) found a decrease of 21.4 per cent in PAH clearance during 45 min supine exercise between period I (0–15 min) and period III (31–45 min) and 16.7 per cent decrease if period III is compared with the mean for periods I and II. With a greater thermal load such as exercising in a hot and humid environment the reduction of hepatic flow is greater at the same relative work intensity (Rowell et al 1965).

#### *Heart rate and stroke volume*

With a constant cardiac output a rise in heart rate indicates a corresponding decrease in stroke volume. The highly significant correlation with a high coefficient of correlation between the increase in heart rate and in body temperature during exercise may indicate a causal relationship between the two variables. A study by Christensen (1931) precludes the possibility of such a direct relation because he had subjects who performed prolonged exercise at steady state in respect of heart rate but with an increase of body temperature and, on the other hand, subjects exercising at a constant body temperature and increasing heart rate. These observations are confirmed by Holmgren's (1956) study of 30 min exercise in well trained men. Instead of a direct relation, the correlation indicates the relation both of the heart rate increase and of the increase in body temperature to the relative work load, either expressed as pulse rate after 10 min exercise or as per cent of  $W_{170}$ . The steeper slope of heart rate with time at the beginning of exercise than at the end could be



due to the fact that most of the temperature increase takes place during the first 30—40 min of an exercise period. The increase in body temperature during exercise was highly significantly correlated to the relative work load expressed as per cent of  $W_{170}$   $r = 0.78$ . That the total increase in body temperature during exercise is correlated to the relative work load and not to the absolute work load as has been suggested (Nielsen 1938) has been shown recently (Saltun & Hermansen 1966).

## *Mechanical systole*

### Previous investigations

The variation in the duration of mechanical ventricular systole has been extensively studied in man by different methods over a long period. For references see Strandell (1964 a) who by means of phonocardiography studied the variation of mechanical systole at rest and during stepwise increased exercise in a group of young and old men. He found a decrease during exercise which was first linearly related to heart rate, but at high heart rates a significant curvilinearly was recorded with successively lesser decrease of mechanical systole.

### Present investigation

Mechanical systole measured from the brachial artery pressure curve was obtained from the 12 subjects investigated in the sitting position (I, IV). The values for all individuals were obtained at rest and from almost all subjects during the prolonged exercise every 10th minute. From the values at rest and during exercise with the exception of the final values (50 or 60 min exercise) and the corresponding heart frequencies the regression equation for mechanical systole on heart rate was calculated (Fig. 3).

Mechanical systole (c sec)  $= 37.8 - 0.119 \times \text{heart rate (beats/min)}$   $r = -0.93$  (highly significant)  $S.D. = 1.55$   $n = 63$

This equation is almost identical to that calculated by Strandell for mechanical systole during stepwise increased exercise in young healthy males in the sitting position (1964 a). If the final heart rates in study I are entered in the regression equation and a value for the mechanical systole is calculated the actual mechanical systole measured is longer in 5 of the 11 subjects, mean difference for all 6 subjects  $2.03$  c sec which is almost significant (see Fig. 3). The same relation exists if the calculation is based on the second degree polynomial equation given by Strandell (1964 a). If the final values from study IV also in sitting position but working at lower intensity are compared with the values calculated from the regression equation no significant difference is found. In study I the subjects were near exhaustion but in study IV they could have exercised for much longer than one hour.

## Heart volume

### Previous investigations

The variations of the heart volume at rest and during exercise have been studied by many investigators. For surveys of the earlier literature see Liljestrand, Lysholm & Nylin (1938) and Rushmer & Smith (1959).

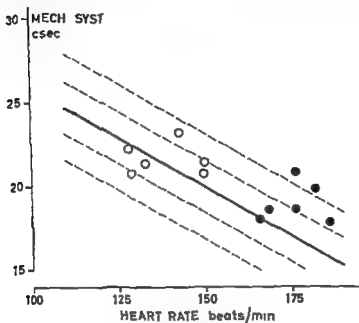


Fig. 3. Regression line  $\pm 2$  S D for mechanical systole on heart rate at rest and during exercise in the sitting position. Open circles are the final values during exercise in study IV. Filled circles are the final values during exercise in study I. For equation see text.

Holmgren & Ovenfors (1960) studied the heart volume in the standing position on transition from rest to exercise in the sitting and supine position and during stepwise increase in work loads both in sitting and supine position. They found that the heart volume was 23.2 per cent smaller after 8 min standing compared with supine position. On the transition from rest to exercise in the supine position there were no significant changes of heart volume. During continued stepwise increased exercise there were no significant changes in the supine position. On transition from rest to exercise in the sitting position the heart volume increased by 10.2 per cent. The heart volume decreased by 6.5 per cent between the first load with an average heart rate of 99 beats/min and the highest load with an average heart rate of 177 beats/min.

### Present investigation

In study V the roentgenological heart volume was measured in 10 healthy young men during prolonged exercise of constant intensity in the sitting position and in 8 subjects also in the supine position. The exposure of the roentgen film was made in the end diastole. During the continuous exercise at a constant load there was a continuous decrease of heart volume in both body positions, accompanied by a continuous increase in heart rate. There was a significant regression of total heart volume on heart rate, with 37.5 ml and 58.3 ml change in heart volume per 10 beats/min change in heart rate in the sitting and the supine positions, respectively. The relative work load was 71 and 67 per cent of  $W_{170}$  in the sitting and supine positions, respectively. Close to exhaustion the heart volume increased in 6 of 10 subjects in the sitting position by, on an average, 52.5 ml and in 3 of 6 in the supine position by, on an average, 42.0 ml, in spite of a further increase in heart rate.

### Comments

*Mechanical systole and heart volume.* The increase in mechanical systole observed near exhaustion during prolonged exercise in relation to the value expected from the regression of mechanical systole on heart rate during stepwise increased exercise seems to be confirmed by preliminary results from further measurements of mechanical systole by phonocardiography (Ekelund & Odar Cederlof 1967). The difference is probably significant even if one takes into consideration the curvilinear part of the regression line at higher pulse rates. This observation might indicate a change in myocardial function during the actual experimental condition, i.e. at a higher intensity of work for about one hour and continued until exhaustion. The increase in final end diastolic heart volume in some of the subjects (V) is probably the same reaction to the same situation as when the mechanical systole increases.

### General discussion

The primary change in the central hemodynamics during prolonged exercise is probably a decrease of the stroke volume, which is compensated for by an increase in the heart rate to keep cardiac output constant. The size of the stroke volume is dependent on the dimensions of the heart and on the emptying and filling of the ventricles. The filling of the ventricles is dependent on the available energy (kinetic and potential) and on the time for filling, and also on the mechanical properties of the myocardium. The filling energy has a static component and a kinetic component. Changes in the distribution of the

blood within the capacity vessels caused for instance, by gravitation will affect the static component and result in a decrease of the stroke volume. The decrease in stroke volume of about the same magnitude both in sitting and supine position during prolonged exercise of the same relative intensity speaks against gravitation as an important factor in the present material. An estimation of the true pulmonary blood volume during prolonged exercise would have been of interest, but the validity of the methods has not yet been confirmed. The delay in a catheter sampling system when using dye dilution methods introduces a large error which could only be corrected for if the transfer function is known so the simple subtraction of delay in seconds is unsatisfactory. The calculation of the transfer function and correction could be performed with the aid of a digital computer (Scheel Langill & Milhorn 1966).

A decrease of the blood volume in the capacity vessels might also be caused by a decrease in the total blood volume. The total blood volume measured with  $I^{131}$  albumin also decreases but the decrease occurs on transition from rest to exercise and during the prolonged exercise the blood volume is unchanged. That a blood volume decrease of the order of 10 per cent increases the pulse rate during exercise has been shown by Gullbring et al (1960) after withdrawal of whole blood and by Danzinger & Cumming (1964) who diminished the plasma volume by 21 per cent in normal subjects with the aid of a diuretic substance. The shift in blood due to vasodilatation caused by the heat regulating mechanism has probably no primary influence on the stroke volume as discussed earlier under the used experimental conditions.

With the decrease of arterial mean pressure there will also be some chronotropic influence from the baroreceptor system with an acceleration of the heart rate resulting in a shorter filling period. Influences from the baroreceptor system or from the central nervous system may also have an inotropic effect on the heart muscle with a change in the myocardial function. For a certain level of work there is probably an optimal combination of filling energy and filling time. A change in one of these components will change the steady state to another level of optimal combination.

The end diastolic dimension of the heart as measured in study V decreases when the heart rate increases. During the same period the mechanical systole decreases linearly in relation to the increase in heart rate with the same regression on heart rate as Strandell (1964 a) found during stepwise increased exercise in healthy young men. Those two findings probably indicate that the degree of emptying of the heart is not affected during the first part of the prolonged exercise. In 6 of 10 individuals in sitting position and in 3 of 6 in supine position the end diastolic heart volume increases shortly before finishing the exercise when the subjects are almost exhausted. In study I the final values

for mechanical systole are probably significantly higher than the values predicted from the corresponding heart rates and the regression equation of mechanical systole on heart rate. On the other hand the final values from study IV which was of a lesser degree of intensity fell within the 95 per cent confidence interval of the regression equation of mechanical systole on heart rate. These two findings might imply that in some individuals at the end of an exhausting exercise period there may be a change of the mechanical properties of the myocardium accompanied by a less effective emptying of the ventricles.

The observed decrease in the systemic arterial mean pressure agrees with earlier observations by Holmgren (1956) and is significant in each study, but is of greatest magnitude during the exercise period at the highest relative work load. The decrease would have been of still greater magnitude if the final values had been compared with pressures obtained after 2 minutes' exercise because we know from earlier studies (Holmgren 1956, Grimby Nilson & Sanne 1966) that there is also a decrease in arterial mean pressure during the first 10 minutes of exercise. In all three studies (I-III-IV) the decrease in brachial arterial mean pressure is partly due to a decrease in the systemic vascular resistance. Between 10 minutes and the end of exercise the decrease is not significant in each separate study but if the three materials are lumped together it is significant ( $P < 0.01$ ). The decrease in the brachial artery mean pressure and the systemic vascular resistance is probably due to a decrease in the vasomotor tone. Another explanation could be a passive dilatation but the long standing decrease in the brachial artery mean pressure reported by Widimsky, Berglund & Malmberg (1963) does not support this assumption.

In all three studies (I-III-IV) there was a decrease in the pulmonary artery mean pressure and also of the calculated pulmonary vascular resistance in the cases when also the pulmonary artery wedge pressure was recorded. The total pulmonary vascular resistance consists of a network of resistances in parallel and series functioning in a non linear mode. The change of alveolar and intra-pulmonary pressure may change the transmural pressure in the pulmonary vessels so that they will pass through the point of critical closing pressure (Permutt & Riley 1963). Such a mechanism will probably operate mostly at the beginning of exercise when the increase in ventilation takes place. There was no correlation between change of pulmonary artery mean pressure and of ventilation or respiratory rate.

The pressure increase on transition from rest to exercise changes the distribution of blood flow so that the pulmonary vascular bed is more evenly perfused especially in the sitting position (West 1962, Holley et al 1966). Such an opening of the inactive vascular bed may result in a decrease in pulmonary arterial mean pressure on transition from rest to exercise. During continuous

exercise however, there is likely to be a more uneven distribution of the perfusion compared with the situation at the beginning of exercise due to an altered distribution of blood within the capacity vessels. Such a mechanism would counteract a decrease in pulmonary arterial mean pressure. Another factor which may change the pulmonary vascular resistance is a change of the diameters of the perfused vessels, which may be rendered passive by the higher pressure during exercise or active due to a change in the vasomotor tone. The passive dilatation of the pulmonary vessels might occur at the beginning of exercise but will probably remain unchanged during prolonged exercise. Therefore a change in vasomotor tone seems to be the factor which causes the fall in the pulmonary arterial mean pressure during continuous prolonged exercise. The long standing decrease in pulmonary arterial mean pressure after a period of exercise observed by Widimsky et al (1963) supports the latter theory. The different distribution of perfusion of the lung vessels in supine and sitting position might be one explanation of the marked difference between the decrease in pulmonary artery mean pressure in the two studies (III-IV) which came earlier in supine than in sitting position. The difference of sympathetic influence might be another factor which alters the pressure response in the sitting compared with the supine position because the sitting or standing position strongly activates the sympathetic nervous system (Vendsalu 1960).

The influence of exercise on the capacity vessels in the arms has been studied by Bevegard & Shepherd (1965-1966). They studied short time supine exercise and found an immediate constriction of the capacity vessels which persisted during 5 min exercise and was related to the work load. Whether such a constriction persists during prolonged exercise is not known from any direct measurements but from the discussion given earlier it seems rather obvious that the tone in the central capacity vessels diminishes during prolonged exercise.

## CHAPTER IV

# Adaptation of the respiration during prolonged exercise

### Previous investigations

Christensen & Hansen (1939) studied three normal, healthy males during prolonged exercise for 2–3 hours. The main interest was to study the change in respiratory quotient during exercise. They found during the prolonged exercise an increase in oxygen uptake in all three individuals, mean 11.1 per cent. They published no figures of total ventilation, alveolar ventilation or respiratory rate. In a study by Levy, Tabakin & Hansson (1961) in which cardiac output in normal men during steady state exercise was measured by a dye dilution technique, 12 males worked on a treadmill for 20 minutes. The authors found no change in total ventilation between 5 min and 12 min work. Davies et al (1965) studied the ventilatory and metabolic response to graduated and prolonged exercise in normal subjects. Three normal subjects worked in sitting position for one hour at 600 kpm/min. There was a slight increase in total ventilation but no figures were given of oxygen uptake.  $P_{CO}$  was about 3–4 mm Hg higher during the first 10 minutes of exercise compared with rest and the last period of exercise.  $P_{CO}$  was however estimated by the Astrup technique and not corrected for the difference between body temperature and the temperature of the pH electrode. In a study by Cobb & Johnson (1963) of prolonged treadmill exercise only an average value for oxygen uptake and ventilation was given.

### Present investigation

In all four studies (I–IV) in which oxygen uptake was measured, there was an increase during the exercise period; this was most marked in the two studies with the highest relative working intensity (I and II), in which the increase was 7.9 per cent and 11.5 per cent respectively. In the two other studies the increase was of the order of 3–5 per cent. In studies I and II there was a significant increase in ventilation of the order of 18.7 and 19.2 per cent respectively. There was also an increase in alveolar ventilation which, however, was of the same magnitude as the increase in oxygen uptake, 10.0 per cent and 13.4 per cent respectively, so that the increased ventilation must also have been caused

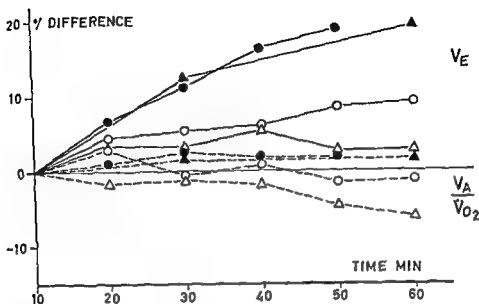


Fig 4 Response of minute ventilation  $V_E$  (—) and alveolar ventilation in relation to oxygen uptake  $V_A/V_{O_2}$  (---) during prolonged exercise. For further data see fig 1. Filled triangles denote values from study II.

by an increase in dead space ventilation (Fig 4). In study I the increased dead space ventilation was caused by an increase both in respiratory rate and in dead space volume, but in study II it was mainly caused by an increased respiratory rate. In the two other studies III and IV there was only a slight but insignificant increase of ventilation. The respiratory quotient RQ increased in all studies on change from rest to exercise. During the continuous prolonged exercise there was a significant decrease with time. The values for RQ during exercise ( $RQ_e$ ) were highly significantly correlated to the value for lactic acid ( $Lact_e$ ) during exercise, the regression equation being

$$RQ_e = 0.841 + 0.0259 \cdot Lact_e \text{ (meq)} \quad n = 103 \quad r = 0.64 \quad S.D. = 0.0323$$

The arterial carbon dioxide tension level was constant during exercise in all four studies (I–IV). The pH of arterial blood decreased slightly in all subjects on change from rest to exercise, then increased slightly during the continuous exercise to about the same value as at rest. Corresponding changes were seen in standard bicarbonate with an initial decrease of 0.8–3.0 meq/litre. The change of acid base status during the work period seemed to be a result mainly of a change in lactic acid.



Lactic acid concentration increased in all cases on change from rest to exercise and then decreased continuously. The same pattern was reported by Bang (1936) from lactate studies during prolonged exercise.

There was no correlation between the increase in ventilation and in heart rate but there was a significant correlation between increase in ventilation and 10 min heart rate and relative work load expressed as per cent of  $W_{170}$ . The increase in minute ventilation and dead space ventilation from 10 min to the end of exercise is highly significantly correlated to the corresponding increase in respiratory rate,  $r = 0.72$  and  $0.86$ . The increase in minute ventilation from 10 min to the end of exercise is also highly significantly correlated to the increase in dead space ventilation during the corresponding period  $r = 0.89$ . The increase in respiratory rate is not correlated to the increase in body temperature between 10 min and end of exercise. This does not confirm the observation of Cotes (1955) who found a relation between body temperature and ventilation during exercise.

In study II the steady state diffusing capacity of the lungs for carbon monoxide ( $DL_{CO}$ ) was measured as well as the other respiratory variables measured in the circulatory studies. Between 7 and 30 min work there was a 12 per cent increase in  $DL_{CO}$  which was probably significant. There was no further change between 30 and 60 minutes. The relative level of work intensity was of the same magnitude as in study I about 75 per cent of  $W_{170}$ . There was a significant increase in ventilation but not of as great magnitude as in study I. The increase in total ventilation was effectuated by an increase in rate of breathing while the tidal volume decreased slightly. This resulted in an increased dead space ventilation. The changes in the physiological dead space were, however, less than those observed in study I.

## Discussion

In the two studies at high working intensity (I and II) there were increases in total ventilation which were due partly to a slight alveolar ventilation corresponding to the slight increase in oxygen. The main increase was caused by an increased dead space ventilation and an increase in respiratory rate. In study I there was also an increase in space volume which might indicate a change in the regional perfusion relationship although the overall ventilation/perfusion ratio ( $V_A/Q_C$ ) changed only slightly. Such a change might indicate a decrease in perfusion of the upper parts of the lung during exercise which could be expected as a result of the increase in arterial pressure. There is also a methodological difference

responsible for the difference in dead space ventilation in studies I and II. In study I the Astrup technique was used for measuring  $P_{aCO_2}$  with a correction of +3.0 mm Hg to make the values comparable with the electrode values for  $P_{CO_2}$ . In the individual case, however, the exact value of the correction factor is unknown and small deviations in  $P_{aCO_2}$  cause rather big changes in dead space ventilation (Holmgren 1965a). In studies III and IV there were no significant changes in total ventilation but in the whole material the change in ventilation and respiratory rate was significantly correlated to the relative work intensity but not to the increase in body temperature as has been suggested by Cotes (1955). The increase in total ventilation is mainly caused by an increase in respiratory rate. The cause of the variation in respiratory rate is however, not quite clear. A stimulation via peripheral chemoreceptors can be excluded because the level of  $PO_2$ , pH and  $P_{CO_2}$  in arterial blood is too stable. The change of body temperature does not seem to be of significance in these studies. The baroreceptor system, especially in the pulmonary vessels, might be responsible for some of the changes in ventilation during the prolonged exercise, i.e. increase of respiratory rate (Kinnison et al. 1965). That exercise increases the level of catecholamines in blood has been shown by von Euler & Hellner (1952), by Holmgren (1956) from studies of urinary excretion of catecholamines after exercise and by Vendsalu (1960) from analyses of blood. In a metabolic study by Carlsten et al. (1965) six young healthy men exercised for 30 min in supine position. They found a progressive increase in norepinephrine level in four of the six subjects. Intravenously infused norepinephrine increases the ventilation mainly through the increase in oxygen consumption (Havel et al. 1964 b, Lundholm & Svedmyr 1966). A progressively increased level of catecholamines in blood could therefore be responsible for at least part of the increase in oxygen consumption and ventilation. A further explanation is a direct central action as a response to the circulatory changes which Gullbring et al. (1960) suggested as being a cause of the increase in ventilation after bleeding. A change of the mechanical properties of the lungs as a result of a diminished pulmonary blood volume might be responsible for some of the changes in ventilation during the prolonged exercise. The slight change in diffusing capacity during the first part of exercise may be a result of opening of unperfused vessels, a mechanism already discussed as responsible for the changes in pulmonary artery mean pressure. The initial increase of diffusing capacity and then of the steady level imply that the prolonged exercise does not decrease but rather increases the capillary blood volume.

## CHAPTER V

### General summary and conclusions

The present investigation is concerned with the circulatory and respiratory adaptation in young healthy men during prolonged exercise of up to one hour on a bicycle ergometer in the sitting and supine position

#### *Total blood volume*

In 18 subjects total blood volume was determined with  $I^{131}$  albumin at rest and at the beginning and end of prolonged exercise both in sitting and supine position. On transition from rest to exercise there was a decrease of 5.0–9.3 per cent depending on the body position and the relative working intensity. During the continuous prolonged exercise the total blood volume and plasma volume were unchanged in all studies.

#### *Central circulation*

Cardiac output was maintained constant in the different body positions and at different relative working intensities. There was a continuous increase in heart rate with a corresponding decrease in stroke volume during the continuous prolonged exercise. The decrease in stroke volume was most marked in the study with the highest relative working intensity (I) but there were no differences between prolonged exercise in supine (III) or sitting position (IV) at the same relative intensity. There was no significant correlation between the decrease in stroke volume during prolonged exercise and the change in minute ventilation, respiratory rate, lactic acid concentration after 10 min exercise or decrease in blood volume from rest to 10 min exercise.

During the continuous prolonged exercise there was a decrease of the pressures both in the systemic circulation and in the pulmonary circulation. The decrease in the systemic circulation was related to the relative working intensity and was caused by a decrease of the peripheral resistance and also by a decrease of the right atrial pressure. The decrease of mean pressure in the pulmonary artery was more marked during supine (III) than during sitting exercise (I–IV) even at a higher relative work intensity.

In the two studies (I, IV) in which the mechanical systole was measured from brachial artery pressure curves both at rest and during the prolonged exercise there was a highly significant regression of mechanical systole on heart rate. The final values of mechanical systole were not included in the calculation of the regression equation. The final values from the study with the highest working intensity (I) were probably significantly larger than the values calculated from the corresponding heart rates.

The end diastolic heart volume measured roentgenologically decreased during the continuous prolonged exercise with a correlation between heart volume and heart rate. In 6 of 10 subjects in the sitting position there was a probably significant increase of heart volume immediately before the end of exercise when the subjects were near exhaustion. The corresponding figures in supine position were 3 of 6 subjects.

The reason for the decrease in stroke volume, central and systemic pressures and in end diastolic heart volume is discussed in connection with the deviation of mechanical systole from the calculated values at the end of exercise to exhaustion. The findings imply that there is a less effective filling of the heart, due to changes in the available filling energy, probably as a result of a decrease in the vasomotor tone. At the end of exercise to exhaustion the results imply that there might also be a change in the mechanical properties of the myocardium, as indicated by the changes of mechanical systole and end diastolic heart volume at the end of exercise.

### *Respiration*

In 3 groups of 6 subjects with different body positions and working intensities there was a continuous increase in oxygen uptake, which caused a corresponding increase in alveolar ventilation. Besides, there was a further increase in total ventilation as a result of an increased dead space ventilation. The increased dead space ventilation was mainly caused by an increase in respiratory rate, but in some studies also by an increase in dead space volume which might be a result of a change in the local ventilation-perfusion relationship. The increased respiratory rate might be a result of reflexes from the baroreceptor system deriving from the receptors in the lung vessels or of an increase of the catecholamine level in the blood. The arterial carbon dioxide tension was unchanged during the prolonged exercise. The respiratory quotient decreased, as did the lactic acid in arterial blood, followed by small changes in pH and standard bicarbonate.

In the 6 subjects performing sitting prolonged exercise the steady state diffusing capacity for carbon monoxide was measured (II) at the beginning

- DAVIES H N, GAZETOPOULOS and C OLIVER Ventulatory and metabolic response to graduated and prolonged exercise in normal subjects *Clin Science* 1965 29 443—457
- EKELUND L G and A HOLMGREN Circulatory and respiratory adaptation during long term non steady state exercise in the sitting position Preliminary report given at the Meeting of the Swedish Society for Clinical Physiology Stockholm Nov. 1962
- EKELUND L G Determination of blood volume *Scand J clin Lab Invest* 1965 17 Suppl 86
- EKELUND L G and I ODAR CEDERLOF Mechanical systole at rest and during stepwise increased exercise and during constant prolonged exercise in the sitting position To be published
- EULER U S v and H HELLNER Excretion of noradrenaline and adrenaline in muscular work *Acta physiol scand* 1952 26 183—191
- FRICKE G Über das Verhalten des Zellfaktors bei körperlicher Arbeit Bestimmungen mit T 1824 (Evansblau) und radioaktivem Chromat *Cardiologia* 1965 47 25—44
- GRIMBY G Renal clearances during prolonged supine exercise at different loads *J appl Physiol* 1965 20 1294—1298
- GRIMBY G N J NILSSON and H SÄRNE Repeated serial determination of cardiac output during 30 min exercise *J appl Physiol* 1966 21 1750—1756
- GULLBRING H A HOLMGREN T SJÖSTRAND and T STRANDELL The effect of blood volume variations on the pulse rate in supine and upright positions and during exercise *Acta physiol scand* 1960 50 62—71
- HALD A *Statistical theory with engineering applications* John Wiley & Sons New York 1960
- HAVEL R J L A CARLSSON L G EKELUND and A HOLMGREN Turnover rate and oxidation of different free fatty acids in man during exercise *J appl Physiol* 1964a 19 613—618
- HAVEL R J L A CARLSSON L G EKELUND and A HOLMGREN Studies on the relation between mobilization of free fatty acids and energy metabolism in man Effects of Norepinephrine and Nicotinic acid *Metabolism* 1964 b 13 1402—1412
- HELLSTRÖM R. and A HOLMGREN On the repeatability of submaximal work tests and the influence of body position on heart rate during exercise at submaximal work loads *Scand J clin Lab Invest* 1966 18 479—485
- HOLLEY H S A DAWSON A C BRYAN J MILIC EMILI and D V BATES Effect of oxygen on the regional distribution of ventilation and perfusion in the lung *Canad J Physiol and Pharmacol* 1966 44 89—93
- HOLMGREN A and K H MATTSSON A new ergometer with constant work load at varying pedalling rate *Scand J clin Lab Invest* 1954 6 137—140
- HOLMGREN A Circulatory changes during muscular work in man With special reference to arterial and central venous pressures in the systemic circulation *Scand J clin Lab Invest* 1956 8 Suppl 24 1—97
- HOLMGREN A and B PERROW Spectrophotometric measurement of oxygen saturation of blood in the determination of cardiac output A comparison with the van Slyke method *Scand J clin Lab Invest* 1959 11 143—149
- HOLMGREN A and C O OVENFORS Heart volume at rest and during muscular work in the supine and in the sitting position *Acta med scand* 1960 167 267—277
- HOLMGREN A and B PERROW The reproducibility of cardiac output determination by the direct Fick method during muscular work *Scand J clin Lab Invest* 1960 12 224—227
- HOLMGREN A B JONSSON and T SJÖSTRAND Circulatory data in normal subjects at rest and during exercise in recumbent position with special reference to stroke volume at different work intensities *Acta physiol scand* 1960 49 313—363

- HOLMGREN A and I STRANDELL On the use of chest head leads for recording of electrocardiogram during exercise *Acta med scand* 1961 169 57-62
- HOLMGREN A and M McILROY Effect of temperature on arterial blood gas tensions and pH during exercise *J appl Physiol* 1964 19 243-245
- HOLMGREN A On the significance of validity and precision in the determination of alveolar carbon dioxide tension for measurements of alveolar ventilation and  $DL_{CO}$  *Scand J clin Lab Invest* 1965 a 17 123-129
- HOLMGREN A On the reproducibility of steady state  $DL_{CO}$  measurements during exercise in man *Scand J clin Lab Invest* 1965 b 17 110-116
- HOLMGREN A and L G EKLUND Central hemodynamics during exercise *Circulat Res* 1967 In print
- HJLTMAN E Blood circulation in the liver under physiological and pathological conditions *Scand J clin Lab Invest* 1966 18 Suppl 92 27-41
- IMBEKTEZ B Jr and K RODAHL Respiratory quotient during exercise *J appl Physiol* 1961 16 606-610
- KENYONOV G L, C J BREZDEN R M CARMACK B M BALLARD P J MEL and A HEMINGWAY Reflex changes in heart rate and ventilation induced by central blood pressure changes *Am J Physiol* 1965 208 1222-1230
- KJELLBERG S R H LOVROTH and U RUDHE The effect of various factors on the roentgenological determination of the cardiac volume *Acta Radiol* 1951 35 413-427
- KROGH A and J LINDHARD The relative value of fat and carbohydrate as sources of muscular energy *Biochem J* 1920 14 290-363
- LARSSON H and S R KJELLBERG Roentgenological heart volume determination with special regard to pulse rate and the position of the body *Acta radiol* (Sthlm) 1948 29 159-177
- LEVY A M B S TABAKIN and J S HANSON Cardiac output in normal men during steady state exercise utilizing dye dilution technique *Brit Heart J* 1961 23 425-437
- LILJESTRAND G E LYSFOLM and G NYLIN The immediate effects of muscular work on the stroke and heart volume in man *Scand Arch f Physiol* 1938 80 265-282
- LUNDHOLM L and N SVEDMYR Studies on the stimulating effects of adrenaline and noradrenaline on respiration in man *Acta physiol scand* 1966 67 63-73
- NIELSEN M Die Regulation der Korpertemperatur bei Muskelarbeit *Scand Arch f Physiol* 1938 79 193-230
- NYLIN G The effect of heavy muscular work on the volume of circulating red corpuscles in man *Am J Physiol* 1947 149 180-184
- PERMUTT B and R L RILEY Hemodynamics of collapsible vessels with tonal waterfall *J appl Physiol* 1963 18 924-937
- ROWELL L B J R BLACKMON R H MARTIN J A MAZZABELLA and R A BRICE Hepatic clearance of indocyanine green in man under thermal and exercise stresses *J appl Physiol* 1965 20 384-394
- RUSHMER R F and O A SMITH Cardiac control *Physiol Rev* 1959 39 41-68
- SALTIN B and J STENBERG Circulatory response to prolonged severe exercise *J appl Physiol* 1964 19 833-838
- SALTIN B and L HERMANSEN Esophageal, rectal and muscle temperature during exercise *J appl Physiol* 1966 21 1757-1767
- SANCETTA S M and L RABITA Response of pulmonary artery pressure and total pulmonary resistance of untrained convalescent man to prolonged mild steady state exercise *J clin Invest* 1967 36 1138-1149

- SCHIEL K W A W LANGILL and H T MILHORN Correction of catheter distortion effects on mean transit time — dye dilution method *J appl Physiol* 1966 21 1637—1641
- SEVERINGHAUS J W and A F BRADLEY Electrodes for blood  $P_{O_2}$  and  $P_{CO_2}$  determination *J appl Physiol* 1958 13 515—520
- SJOSTRAND T A method for the determination of the total haemoglobin content of the body *Acta physiol scand* 1948 16 211—231
- SJOSTRAND T *Functional capacity and exercise tolerance in patients with impaired cardiovascular function* Clinical cardiopulmonary physiology Grune & Stratton Inc New York 1960
- SNEDECOR G W *Statistical methods* Iowa State College Press Iowa 1959
- STRANDELL T Mechanical systole at rest during and after exercise in supine and sitting position in young and old men *Acta physiol scand* 1964 a 61 279—298
- STRANDELL T Heart rate arterial lactate concentration and oxygen uptake during exercise in old men compared with young men *Acta physiol scand* 1964 b 60 197—216
- STROM G The influence of anaemia on lactate utilization in man after prolonged muscular work *Acta physiol scand* 1949 17 440—451
- THOMPSON W O P K THOMPSON and M E DAILEY The effect of posture upon the composition and volume of the blood in man *J clin Invest* 1928 5 573—604
- TORNVALL G Assessment of physical capabilities *Acta physiol scand* 1963 58 Suppl 201
- UEHLINGER A and A BUHLMANN Das Verhalten des Blutvolumens während kurzfristiger körperlicher Arbeit *Cardiologia* 1961 38 357—370
- VENDSALU A Studies on adrenaline and noradrenaline in human plasma *Acta physiol scand* 1960 49 Suppl 173
- WADE O L and J M BISHOP Cardiac output and regional blood flow Oxford Blackwell scientific publications 1962
- WEST J B Regional differences in gas exchange in the lung of erect man *J appl Physiol* 1962 17 893—898
- WIDIMSKY J H BERGLUND and R MALMBERG Effect of repeated exercise on the laser circulation *J appl Physiol* 1963 18 983—986











ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 294

From the Institute of Physiology University of Lund, Sweden

FACTORS OF IMPORTANCE  
FOR THE ETIOLOGY OF  
OBESITY IN MICE

By

STIG LA

LUND



ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 294

From the Institute of Physiology, University of Lund Sweden

FACTORS OF IMPORTANCE  
FOR THE ETIOLOGY OF  
OBESITY IN MICE

By

STIG LARSSON

LUND 1967



## CONTENTS

Introduction	5
Chapter I General material and methods	7
Chapter II, Some characteristics of Swiss, NMRI and CBA mice	15
Chapter III Influence of protein content and quality in the food on growth body fat content and food efficiency in various strains of mice	25
Chapter IV, The effect of a diet composed of the average 1960 consumption in Sweden compared with a diet suggested by the National Institute of Public Health and with a third of low carbohydrate and high fat content, on growth body fat content and protein fat ratio in two strains of mice	33
Chapter V, Production of goldthiogluucose obesity in different strains of mice	41
Chapter VI The effect of muscular activity on fat mobilization and body fat content in normal and obese mice	49
Chapter VII Eating behaviour in normal and obese Swiss, NMRI and CBA mice	59
Chapter VIII, Food preferences in different strains of normal and obese mice	65
General conclusions and summary	75
References	78





## INTRODUCTION

The mechanism of regulation of food intake is complicated and still not completely understood (cf Andersson and Larsson 1961, Mayer 1964, and Larsson 1967). Even if many factors are known to influence the feeding mechanisms the nature of the adequate stimulus is not known. As a result of the last fifteen years of research in this field, however it has become increasingly obvious that the adequate stimulus for the "feeding center" is of multiple origin.

It is natural that the nutritional state is dependent on the integrative actions of the feeding mechanisms. In this connection the classical experiments on the importance of an intact hypothalamus for the nutritional homeostasis made by Brobeck and coworkers deserve attention (cf Brobeck 1955). Thus a prolonged state of hyperphagia caused by bilateral lesions in the ventromedial parts of the hypothalamus leads to overnutrition which in general results in obesity, almost regardless of the composition of the diet at least as long as it fulfills the basic caloric needs (cf Brobeck 1955).

Many factors have been found to influence the quantity of the food intake. It is generally accepted that under physiological conditions energy intake and expenditure are equal. In this connection the quality of the food eaten has to be considered. The normal individual keeping constant weight and largely constant body composition will have the ability to adjust the amount of food eaten depending on the quality with regard to protein, fat and carbohydrate. The accuracy with which nutritional homeostasis is maintained will vary from individual to individual, but will also vary due to the external environment. Therefore in order to evaluate the cause of a changed nutritional state hereditary factors must be considered. In recent years much interest has been focused on the hereditary obesity in laboratory animals (cf Mayer 1964, and Hellman 1965). In animal husbandry the nutritional state of the animals is of fundamental importance and here both hereditary and external factors have to be considered. Also in dogs it has been found that hereditary factors are of importance for the etiology of obesity and related diseases (Brook, Larsson and Rooney 1960).

Mayer (1964) differs between regulatory and metabolic obesity, well aware of the fact that such a distinction, although usefull necessitates a certain degree of over simplification. It still leaves the question open whether a disturbance in the regulatory mechanism leading to over nutrition will not change the general metabolic pattern so that a metabolic type could be considered.

Other factors such as exercise have the ability of influencing food intake (cf Mayer 1964, Kennedy 1964 and Parizkova 1966). Also a change of diet in laboratory and domestic animals affects food intake and has a profound influence on the body composition (Ericson, Larsson and Rubarth 1962b, Clausen 1963, Munck 1964 and Larsson 1966a). A laboratory animal when offered a free selection of diet instead of the ordinary laboratory diet might also change in body weight (Larsson 1957). In hereditary obesity in mice it has been found that different diets have profound effects on the development of obesity (Mayer 1964).

The age of the animal has also importance regarding the fat content of the body as well as the fat mobilizing capacity (Benjamin *et al* 1961, Altschuler, Lieberson and Spitzer 1962, and Jelinkova and Hruza 1964).

Thus many factors have been found to exert influence upon the nutritional fate of the organism. As has been pointed out, the feeding mechanisms *per se* cannot be studied and discussed separately without taking into account the contribution of many factors which acting together, maintain a nutritional homeostasis in the normal individual (Larsson 1967).

The present study was made with the purpose of comparing the hereditary factors in various strains of mice in relation to external factors such as exercise and different types of diets for the etiology of obesity.

# CHAPTER I

## GENERAL MATERIAL AND METHODS

In the present study mice from three different strains were used *viz* the Swiss NMRI and CBA strains, which were originally obtained from the Institute of Genetics (L. Munck) University of Lund, and then bred at the Institute of Physiology

Except when otherwise stated, the animals were fed *ad libitum* and given a pellet diet. The composition of the food may be seen in Table I A and B. The mixing and pelleting of the food was done by AB Teknosan Malmö. The food was bacteriologically controlled and stored at  $+4^{\circ}\text{C}$ .

The mice had free access to tap water. The animals used for breeding received in addition to the standard diet an extra supply of calcium phosphorus and vitamins. The litters were weaned when 22 days old.

The animals were generally kept in macrolon cages with wood shavings. When the mice were starved not only the food but also the wood shavings were taken away.

The size of the cages varied according to the experimental set up and will be given for each experiment. If not otherwise stated the animals were weighed once a week.

Experimental obesity in the various strains of mice was produced by a single intraperitoneal injection of goldthioglucose (Solganol II oleosum Schering). To obtain maximal efficiency of the injections with regard to the development of obesity the Swiss mice were usually injected in the fed state with 0.8 mg goldthioglucose per g body weight. The NMRI and CBA mice were injected after 24 h fast with 1.5 mg and 0.5 mg per g body weight respectively. The reason for the different doses used will be discussed in Chapter V.

When blood samples were taken the technique described by Rerup and Lundquist (1966) was applied. This method is based on orbital bleeding by means of constricting pipettes and allows daily blood sampling of at least 25  $\mu\text{l}$  without upsetting the basic physiological conditions. It is furthermore a very rapid way of blood sampling.

TABLE I A

The composition of the standard food used in the experiments

Content per kg food			
	g		mg
Barley	400	Vit B	20
Oats	120	Vit B <sub>6</sub>	5
Wheat	100	Nicotylamide	40
Lucerne	30	Folic acid	0.2
Meat meal	50	Calcium pantothenate	60
Fish meal	60	Choline bitartrate	40
Dried skim milk	34	Vit A	30 000 IU
Dried pig blood plasma	10	Vit D	3 000 IU
Casein	10	Vit E	70
L lysine HCl	15	Ferrosulphate	500
DL methionine	0.5	Manganese sulphate	200
Lard	6	Copper sulphate	175
Glucose	10	Cobalt sulphate	60
Dried brewer's yeast <sup>1)</sup>	25	Zinc sulphate	50
		Magnesium sulphate	100
		Sodium chloride	5000

<sup>1)</sup> Composition of the dried brewer's yeast: Content in %: Protein 50, carbohydrates 35, ash 8 and water 7.

Vitamin content ( $\mu\text{g/g}$ ): B<sub>1</sub> 120–150, B<sub>2</sub> 30–50, Nicotinic acid 400, Pyridoxine 50, Folic acid 30, B<sub>12</sub> not known, Pantothenic acid 200, Choline 3500, Inositol 3000, Biotin 1 and Para aminobenzoic acid 10.

TABLE I B

General characteristics of the standard food used in the experiments (in %)

Raw protein	23.7	Digestible protein (determined with pepsin and HCl)	18.6
Raw fat	4.0	Non digestible protein	1.5
Nitrogen free extractable substances	50.6	Amides	3.6
Crude fibre	4.5	Calcium	1.36
Ash	7.5	Phosphorus	1.08
Water	9.7		
	100.0		

### Incubation of adipose tissue *in vitro*

In order to study the net release of glycerol and free fatty acids (FFA) from adipose tissue *in vitro* the following general procedure was followed:

The mice were killed by cervical dislocation. About 50 mg from each side of the epididymal fat pad was carefully removed.

The fat was incubated in a Dubnoff type metabolic shaker at 37°C in Hagerdorn tubes for one hour with and without adrenaline (1  $\mu$ g/ml) in 4 ml Krebs Ringer phosphate buffer to which was added 2 % albumin (bovine serum albumin Sigma). The incubation was carried out in air. The pH of the incubation fluid at room temperature was 7.40–7.43. The ionic concentration of the buffer was the same as that used by Munkner (1963), containing NaCl 120 mMol/l, KCl 5.6 mMol/l, CaCl<sub>2</sub> 2.6 mMol/l, KH<sub>2</sub>PO<sub>4</sub> 1.2 mMol/l, MgSO<sub>4</sub> 1.3 mMol/l and Na<sub>2</sub>HPO<sub>4</sub> 10 mMol/l.

Particularly in studies concerned with the inhibition of lipid mobilization *in vitro* bicarbonate buffer is as a rule preferred to phosphate medium. Thus Martin, Horning and Vagelos (1961) have found that bicarbonate ions are essential for normal lipid synthesis.

When bicarbonate buffer was used and the results of the release of glycerol and FFA compared with phosphate medium no differences were observed.

A small portion of the gonadal fat was taken out from the mice and immediately frozen for further analysis on the content of glycerol and FFA.

After incubation the adipose tissue was cleaned in ice cold isotonic sodium chloride and frozen. The incubation fluid was also frozen.

In each experiment the albumin containing medium was analyzed for FFA. As a rule no glucose was added to the medium.

Addition of glucose to the medium has been found to reduce the release of FFA (Lopez, White and Engel 1959 and Raben and Hollenberg 1960). Since the total FFA content of the system decreases, one must assume an enhanced esterification. Further, glucose in the medium will not cause any change in the release of free glycerol (Vaughan 1962).

#### *Determination of FFA in blood plasma, incubation medium and adipose tissue*

The method used was essentially the same as that published by Laurell and Tibbling (1967). Thus 0.1 ml plasma, 0.5 ml medium or 0.2 ml homogenized adipose tissue (1:20) were shaken with 1 ml 1/15 M phosphate buffer pH 6.8 and 6 ml chloroform—heptane (4:3 v/v). After centrifugation the upper phase was discarded. The chloroform—heptane phase was shaken with an alkaline coppernitrate/ethanolamine solution saturated with NaCl in order to obtain the chloroform—heptane mixture in the upper

phase The FFA in this phase will participate in a FFA copper complex in proportion to the FFA concentration Part of the upper phase is used together with diphenylcarbazide which forms a redish copper complex, the extinction of which is measured at 540m $\mu$  Palmitic acid is used as the standard This method gives good reproducibility and seems to be more sensitive and specific than other methods described in the literature

Thus samples analyzed after this method showed close correlation with samples analyzed when run on silica gel )

The test tubes used in the extraction procedure of the FFA and the pipettes used were silicon treated which seemed necessary in order to get reproducible values

#### *Determination of glycerol in blood plasma incubation medium and adipose tissue*

Glycerol was determined according to Laurell and Tibbling (1966) using an enzymatic fluorometric method allowing measurements of very small amounts

The method is a fluorometric adaptation of the enzymatic conversion of glycerol where reduced diphosphopyridine nucleotide is formed in proportion to the glycerol concentration The samples were read in a spectro-fluorometer at exciting wave-length at 350 m $\mu$  and emitted at 458 m $\mu$  An Aminco Bowman instrument was used for these measurements

Blood sugar was determined by the method by Marks (1959) with 0.33 M perchloric acid buffered with glycine to give pH 2.7, as the protein precipitant

#### *Determination of carcass content of water protein and fat*

After the adipose tissue samples had been taken, the gastro intestinal content was removed by washing and the animals frozen The carcass content of water protein and fat was determined as described previously (Larsson 1966a) Thus the mice were homogenized *in toto* with water in a MSE homogenizer An aliquot of the homogenate was further homo-

---

\* ) These determinations were made by Dr Laurell and Dr Tibbling Department of Clinical Chemistry University of Lund

homogenized and diluted with water for protein analysis according to Lowry *et al* (1951) The total mouse homogenate was dried to obtain the water content of the body employing the calculation formula used by Munck (1964)

$$X = \frac{(I-U)(M+V)-I V}{I M} 100$$

X = water content of carcass in %

V = added water (ml)

M = weight of mouse (g)

I = weight of homogenized animal (g)

U = weight of dry substance (g)

The dried homogenate was then ground and extracted with petroleum-ether in a Soxhlet apparatus for 10 hours to obtain the fat content

### *Biological definition of the standard food*

As may be seen from Table I A and B the protein content of the standard laboratory food generally used in the present study is comparatively high As shown by Ericson Larsson and Lid (1961), however it is not the protein quantity that determines the growth rate and food utilization of a diet Rather it is the protein quality that is largely responsible for the high values of these parameters By measurements of the protein efficiency ratio (PER) and by carcass analysis of the content of fat and protein one can largely judge the quality of the food

For this purpose newly weaned (22 days old) male mice of the CBA strain were given the standard food for three weeks The weight of the

TABLE II

Growth, protein fat ratio and PER in male CBA mice on the standard laboratory food and on a reference cereal diet (>3 animals in each group)

	Growth in g	Protein — fat ratio	PER
Standard lab food	14.5	2.12	2.70
Cereal food	8.7	0.96	1.40

) PER=growth per g protein consumed.



animals was measured once a week as was the food intake. For comparison, a diet containing only the cereal components supplemented with the vitamins and minerals shown in Table I A was fed to another group.

After three weeks on the diets the animals were killed by cervical dislocation and the carcass content of protein, fat and water was determined as described previously in this chapter.

Table II shows the results of this introductory study and reveals that the standard diet gives high growth rate and protein:fat ratio.

### *Expression of data*

All experimental evidence indicates that fat is mobilized from adipose tissue probably entirely in form of FFA. During incubation *in vitro* the fatty acids are released into the medium. However, the fact that the tissue content of FFA may undergo changes suggests that the tissue content of FFA before and after incubation must be of importance to assess the net release of FFA. Thus Vaughan (1962) has found that FFA may appear in the medium as a result of transfer from the tissue FFA compartment, not necessarily paralleling the net generation of FFA in the system. Of importance in this connection is that all albumin preparations contain a significant amount of FFA, unless specially pretreated (Goodman 1957). This requires, of course, that the zero time FFA content will be measured both in the incubation medium and in the adipose tissue.

It is obvious that due to the fact that FFA production can be influenced by changes in the rate of triglyceride synthesis (resynthesis of released FFA) as well as by changes in the rate of lipolysis, true information of lipid mobilization cannot be obtained by using FFA net release as the only parameter (cf. Steinberg and Vaughan 1965).

As studies have shown that free glycerol is metabolized only very slowly by adipose tissue (Shapiro, Chowder and Rose 1957 and Steinberg and Vaughan 1961) due to absence of demonstrable phosphoglycerokinase activity (Wieland and Suyter 1957, and Margolis and Vaughan 1962) measurement of net release of glycerol is considered to be a safer parameter for the fat metabolism *in vitro* (cf. Steinberg and Vaughan 1965). However, changes in the fate of glycerol in adipose tissue must be kept in mind although they are regarded as less important when compared with changes in net release of FFA (cf. Steinberg and Vaughan 1965).

The average reading when 18 samples with 10  $\mu$ M palmitic acid were determined was  $0.623 \pm 0.0044$ , whereas the corresponding figure when the same number was determined on 18 consecutive days was  $\pm 0.0073$ .

The average reading when 20 samples with 0.4 mM glycerol were determined was  $0.220 \pm 0.0027$ , and when the same number determined on consecutive days was  $\pm 0.0050$ .

The problem as to the correct way of expressing the data from the metabolic events of the adipose tissue can be discussed indefinitely. Thus Hellman, Larsson and Westman (1962) pointed out the importance of expressing metabolic data in relation to the number of cells, i.e. metabolic units, and also emphasized the importance of considering the biochemical data with regard to morphological findings. This statement became more evident when it was shown that the number of mast cells greatly increased in adipose tissue in obesity in mice (Hellman, Larsson and Westman 1963). Therefore it was felt wrong to express data in terms of nitrogen or DNA in the present study. Also the expression of data in terms of number of fat cells could be irrelevant since also the mast cells could be regarded as having a metabolic activity of their own. Thus the data in the present study regarding the *in vitro* experiments are expressed as units per g weight although it might be regarded as a certain degree of over simplification.

### *Criteria and definition of obesity*

In the literature there is no general agreement as to the definition of obesity. Therefore the dividing line between leanness and obesity will be arbitrary. Fenton and Dowling (1953) have suggested a criterion of obesity based upon the relative amounts of body fat and of the fat free weight compartment. Thus by plotting carcass fat against fat free weight for individual animals at a certain age one obtains a certain regression line the slope of which eliminates the obese individuals. Such a way of defining obesity — as a marked deviation from the regression line — is no doubt very suitable when the mice are not too old. Many older mice, as will be shown later in this study, however, will develop age obesity.

Figure 1 shows the relation between fat and fat free body weight in the Swiss NMRI and CBA mice. The broken lines represent the regression line plus minus twice the standard error. Above this the animals are regarded as obese.

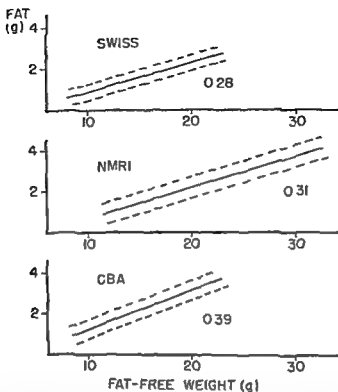


Fig 1 Regression line for fat against fat free weight in Swiss NMRI and CBA mice 22—180 days old. The mice have been given the standard laboratory food. The slope of the line is given in the figure. Broken lines indicate twice the standard error of estimate. In each strain about 200 animals have been used.

## CHAPTER II

"Lack of success in the treatment of obesity requires that we maintain a flexible attitude towards these patients  
*Gordon 1954*

### SOME CHARACTERISTICS OF SWISS, NMRI AND CBA MICE

by

STIG LARSSON and SVEN JONSSON

In line with the preliminary study demonstrating strain differences in mice with regard to decrease in weight, change in body composition and in FFA of blood plasma after 24 hours of fasting (Larsson 1966 b), it seemed appropriate to obtain more information on these differences.

As briefly reviewed in the introduction, many factors are thought to contribute to nutritional homeostasis. The influence of these could, however, be expected to have different importance in various species and strains within a species.

Lyon and Fenton (1956) have reported that the levels of glycogen in liver and muscle differ in various strains of mice in relation to inherited differences in metabolic and endocrine patterns. The feeding of diets with high fat contents can induce obesity in some strains of mice but not in all (cf Fenton 1960). Such breed characteristics have also been observed in dogs with regard to the occurrence of obesity and diabetes mellitus (Brook *et al* 1960).

In the obese state it has been shown that the FFA content of adipose tissue will vary in rise after starvation in different types of obesity in mice (Hollifield, Perlman and Parson 1962). It could, therefore, be possible that in the different strains of mice the glycerol and FFA content in and release from adipose tissue could be different.

The present experiments were made to further examine the observed strain differences with regard to different parameters believed to be of importance for nutritional homeostasis and thus for the etiology of obesity.

### *Material and Methods*

Mice of the Swiss NMRI and CBA strains were used. They were as usual fed *ad libitum* with free access to tap water. The food was the standard diet described in Chapter I.

At the end of the experimental period the animals were killed and analyzed with regard to the carcass content of water, protein and fat as described in Chapter I.

*Experiment 1* From each strain 10 mice were starved for 24 hours at eleven different ages. The weight loss was measured and the animals killed.

by cervical dislocation. Part of the epididymal fat was incubated as described in Chapter I. The same procedure was made in mice in the fed state. Groups of mice from each strain were also starved for 6 hours and treated as above.

*Experiment 2* Goldthioglucose obesity was produced in the Swiss, NMRI and CBA strains as described in Chapter I. The criteria for obesity were the same as outlined in the same chapter. The animals were not used in the experiment proper until they had reached the static phase of obesity, namely, they did not increase appreciably further in weight and/or fat content on the standard laboratory food, after having passed the dynamic phase of obesity. At this time they were on the average 95 days old. Groups of the obese mice were starved for 24 hours and the decrease in weight measured. After killing the animals by cervical dislocation, they were treated as described in Chapter I regarding carcass analysis and the net release *in vitro* of glycerol and FFA from adipose tissue.

*Experiment 3* Mice of the Swiss, NMRI and CBA strains at the ages of 33, 95 and 180 days were used. After 24 h of food deprivation in half of the animals they were subjected to blood sampling as described in Chapter I and the blood analyzed with regard to the content of blood sugar as described in the same chapter.

## Results

*Experiment 1* Figure 2 shows the decrease in weight of mice of different strains at various ages after 24 h deprivation of food. It may be noted that young Swiss mice decrease up to 30 % of the body weight during this time while the NMRI and in particular the CBA mice exhibit a smaller decrease in weight at the same age. In common for all three strains the decrease in weight is less pronounced with increasing age (Figure 2).

The decrease in weight after 24 h of fasting is followed by a marked decrease in body fat content which may be seen in Figure 3, where the fat content is plotted against the fat free weight (see Chapter I). The decrease in body fat largely follows the same characteristics in strain and age as mentioned for the weight decrease. However, new regression lines for the slope after 24 h fast in the various strains can be drawn (cf. Fig. 1).

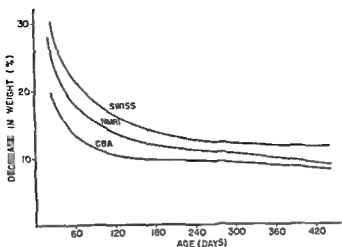


Fig 2 Decrease in weight (%) after 24 h fast in male Swiss NMRI and CBA mice at different ages

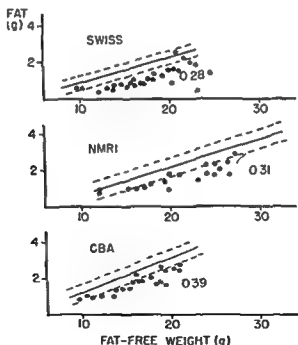


Fig 3 Carcass fat plotted against fat free weight in Swiss NMRI and CBA mice after 24 h fast (solid circles) in relation to corresponding values in the fed state as indicated in Fig 1

Table III shows the content in epididymal fat of glycerol and FFA of mice in the fed state and in those starved for 6 h and 24 h. Figures 4 and 5 others have been expressed in the same way as used by Munkner (1963) for other parameters. As may be seen from the Table and Figures 4 and 5 certain differences exist. Thus, there is a difference in the glycerol content of the adipose tissue in Swiss mice between the values from 24 h fast and those obtained in the fed state and after 6 h fast. The same tendency is met regarding the NMRI and CBA mice (Figure 4). Among the different strains there is only a difference after 24 h fast between Swiss mice and NMRI and CBA mice. As may be seen in Figure 5, the FFA

TABLE III

The content of glycerol and FFA in the epididymal fat pad of male Swiss, NMRI and CBA mice in the fed state, and after 6 and 24 h of fast. The age of the animals is between 90 and 140 days. Mean  $\pm$  s.e.m. ( ) = number of animals. Differences in Fig. 4 and 5.

Strain	Glycerol $\mu$ M/g adipose tissue			FFA $\mu$ M/g adipose tissue		
	Fed	6 h fast	24 h fast	Fed	6 h fast	24 h fast
Swiss	3.3 $\pm$ 0.40 (12)	2.9 $\pm$ 0.37 (7)	8.0 $\pm$ 0.48 (7)	0.7 $\pm$ 0.04 (13)	1.3 $\pm$ 0.10 (9)	2.1 $\pm$ 0.14 (8)
NMRI	4.1 $\pm$ 0.44 (11)	3.6 $\pm$ 0.24 (8)	5.5 $\pm$ 0.29 (10)	0.4 $\pm$ 0.04 (10)	1.0 $\pm$ 0.04 (8)	1.7 $\pm$ 0.07 (8)
CBA	3.6 $\pm$ 0.57 (11)	3.3 $\pm$ 0.29 (8)	5.7 $\pm$ 0.34 (5)	0.3 $\pm$ 0.05 (14)	0.7 $\pm$ 0.04 (10)	1.5 $\pm$ 0.06 (8)

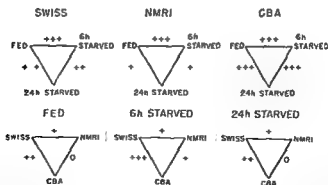


Fig. 4. Differences in glycerol content of epididymal fat pad in male Swiss, NMRI and CBA mice fed and after 6 or 24 h fast, calculated according to Student's *t* test, after the values in Table III. +++ =  $p < 0.001$ , ++ =  $p < 0.01$ , + =  $p < 0.05$  and 0 = no difference.



content in adipose tissue differs significantly in each strain due to the length of food deprivation. Among the three strains there are significant differences except between NMRI and CBA mice in the fed state and after 24 h fast.

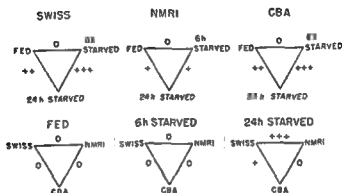


Fig 5 Differences in FFA content of epididymal fat pad in male Swiss, NMRI and CBA mice fed and after 6 or 24 h fast calculated according to Student's *t* test after the values in Table III. +++= $p<0.001$ , ++= $p<0.01$ , += $p<0.05$  and 0=no difference.

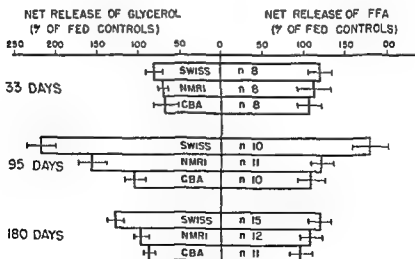


Fig 6 The net release of glycerol and FFA from epididymal adipose tissue in male Swiss, NMRI and CBA mice fasted for 24 h compared with fed animals at three different ages. The differences are calculated  $\pm$ sem in % deviation from the corresponding fed mice.

Figure 6 shows the net release of glycerol and FFA from adipose tissue *in vitro* in mice starved for 24 h from three age groups, compared in percent with the values of fed litter mates. From this figure it may be noted that in the older groups of mice the glycerol values differ significantly while in the 33 days old mice there is no significant difference. Among the various age groups, it may also be seen, that it is only the Swiss mice which have a significantly higher FFA net release compared with the two other strains.

A response to adrenalin was noted but followed the same magnitude, relatively as without this substance.

**Experiment 2** It may be seen from Table IV that starvation of obese Swiss and NMRI for 24 h causes significantly less weight loss than in corresponding lean animals. The 24 h fast increased the glycerol and FFA content in adipose tissue significantly in obese Swiss mice compared with the corresponding fed (obese) animals (Table V).

TABLE IV

The effect of 24 h of fast in male obese Swiss, NMRI and CBA mice with regard to decrease in weight (in % to non fasted animals) compared with corresponding values in fasted non obese mice. Mean  $\pm$  s.e.m. Differences calculated according to Student's t test: a)  $p < 0.001$  b)  $p < 0.01$ .

	Swiss	NMRI	CBA
Controls	18 $\pm$ 0.6 <sup>a</sup>	15 $\pm$ 0.4 <sup>b</sup>	11 $\pm$ 0.3
Obese	14 $\pm$ 0.4	12 $\pm$ 0.5	10 $\pm$ 0.8

TABLE V

The content of glycerol and FFA in the epididymal fat pad of obese fasted and fed Swiss, NMRI and CBA mice. Mean  $\pm$  s.e.m. ( ) = number of animals. Differences calculated according to Student's t test: a)  $p < 0.001$  b)  $p < 0.01$  and c)  $p < 0.05$ .

Strain	Glycerol $\mu$ M/g adipose tissue		FFA $\mu$ M/g adipose tissue	
	Fed	24 h starved	Fed	24 h starved
Swiss	14 $\pm$ 0.05 <sup>b</sup> (13)	20 $\pm$ 0.19 (8)	04 $\pm$ 0.05 <sup>a</sup> (13)	07 $\pm$ 0.05 (8)
NMRI	15 $\pm$ 0.11 <sup>a</sup> (9)	21 $\pm$ 0.18 (9)	03 $\pm$ 0.03 (9)	04 $\pm$ 0.04 (9)
CBA	13 $\pm$ 0.10 (10)	16 $\pm$ 0.12 (9)	03 $\pm$ 0.04 (10)	04 $\pm$ 0.04 (9)

The net release of glycerol and FFA from the adipose tissue *in vitro* due to the starvation for 24 h did not increase significantly in either of the three strains even if there was a tendency towards higher values. The response to adrenalin *in vitro* followed exactly the same pattern.

**Experiment 3** When normal mice were starved for 24 hours there was a significant decrease in the blood sugar values. The decrease was particularly obvious in the Swiss mice at any age, even if changes also took place in the two other strains after starvation (Table VI).

TABLE VI

Plasma glucose of normal male Swiss NMRI and CBA mice at three different ages in the fed state and after 24 h of fast. Mean  $\pm$  s.e.m. 45 animals in each group. Differences calculated between fed and starved animals according to Student's *t* test. a)  $p < 0.001$ .

Strain		Plasma glucose mg/100 ml of blood			
		Age (days)	33	95	180
Swiss	Fed		123±4.8 <sup>a</sup>	129±6.0 <sup>a</sup>	128±5.5 <sup>a</sup>
	Starved		47±2.5	61±3.8	72±3.6
NMRI	Fed		121±6.9 <sup>a</sup>	124±7.2 <sup>a</sup>	126±6.8 <sup>a</sup>
	Starved		82±5.8	91±4.9	90±5.4
CBA	Fed		127±4.9 <sup>a</sup>	129±6.4 <sup>a</sup>	125±4.8 <sup>a</sup>
	Starved		85±5.7	89±7.7	94±6.2

## Discussion

The results obtained from experiment 1 in this chapter clearly indicate strain differences. Without food for 24 hours the young Swiss mice had the heighest weight losses in %. In these animals the 24 hours of fasting also have a profound effect on the blood sugar level which goes down to almost hypoglycaemic levels (Table VI). This finding confirms the previous preliminary experiments (Larsson 1966b). The fat content of the carcasses of the young Swiss mice was extremely low after the 24 h deprivation of food. In the youngest mice it was even impossible to get enough fat for incubation. In a study of the effect of body weight on free fatty acid release by adipose tissue *in vitro* it was found that the youngest most rapidly growing rats released the most FFA per unit weight of tissue (Altschuler *et al* 1962). Further with increasing age the responsiveness of

adipose tissue to adrenalin *in vivo* and *in vitro* decreases as measured by the release of FFA (Benjamin *et al* 1961, Dury 1962 and Jelinkova and Hruza 1964) These findings could not be supported by the present study On the other hand, the mice studied in the present investigation were not as old as the rats used in the previous studies Further, it has been shown that probably the measurements of glycerol is more adequate *in vitro* as *in vivo* (Chapter I)

It can be discussed whether the adipose tissue of obese mice with respect to the metabolism of glycerol and FFA does not behave like adipose tissue in old mice In this respect it is necessary to point upon the results from starvation Thus, Chlouverakis (1962) found that the adipose tissue content of lipoprotein lipase significantly dropped in rats with increasing age In the present study very old mice have not been studied On the other hand the present results indirectly confirms this observation Modern research with a tendency to use radioactive compounds to study metabolism of this sort has a tendency to overlook the possibility to use parameters such as body weight and fat content of the body By using these measurements one will assure more accurate determinations of the total effect of a "physiological situation when studying nutritional homeostasis

The differences due to the strains considered seem to be of importance Cheek and Holt (1963) studied the growth and body composition of male albino mice They found that the weight of the mice when plotted against the age gave an S shaped curve regarding growth The same curve was found when fat and water contents were plotted against age This way of expressing obesity might be very useful but as has been shown it is not the right evaluation of obesity (Larsson 1966a) In certain types of adiposity overweight is not found but a relative increase of body fat with age Such data has been given for animals (Moulton 1923) and for man (Brozek 1952) Other studies in man seem to point in the same direction (Keys 1964) The present results are largely in agreement with the previous findings Between the different strains however marked differences were noted with regard, in first hand to the reaction towards 24 h of fast

Marshall (1961) studying the influence of adrenaline and fasting on FFA mobilization in goldthiogluucose induced obesity found that FFA production was similar in adipose tissue of fasted obese and lean mice This observation is only partly in agreement with the results of the present study Rather the results in this investigation are more like those obtained by Hollifield *et al* (1962) They studied the FFA content of adipose tissue

in three types of obese mice during fasting and found that in goldthio glucose obese mice, the adipose tissue content of FFA was lower than in corresponding controls

When the carcass fat content of the mice starved for 24 h is plotted against the fat free weight one gets the impression that a new regression line can be drawn from that in fed animals as described in Chapter I. The slope will be lower in the Swiss mice than in the two other strains, indicating a more active fat mobilization. As mentioned in the beginning of this discussion this chapter indicates definite strain characteristics regarding the mice of the different strains. It is therefore logical that many non confirming observations from laboratory to laboratory which are not in the same line could depend on this strain phenomenon. The present results show that hereditary factors play an important role for the nutritional fate of the mice in the present study with regard to the deposition of body fat and thus being a factor of importance for the etiology of obesity.

## CHAPTER III

"No pig can form lean meat up to the limit determined by its hereditary unless its diet has sufficient quantities of protein of high biological value and no pig can be forced, by means of extraordinary high levels of protein in its diet to produce more lean meat than permitted by its hereditary

*Clausen 1963*

### INFLUENCE OF PROTEIN CONTENT AND QUALITY IN THE FOOD UPON GROWTH, BODY FAT CONTENT AND FOOD EFFICIENCY IN VARIOUS STRAINS OF MICE

by

STIG LARSSON

In recent years several studies have demonstrated the importance of the protein quality for growth and feed efficiency in animals. By these studies it has become evident that it is not the protein content of the food *per se* that determines the biological value, but rather the optimal participation of essential amino acids (Ericson *et al* 1961 and Munck 1964). The processing of natural proteins often decreases the quality as measured by the growth rate and food utilization due to a decrease in the availability of certain essential amino acids (*cf* Ericson *et al* 1961, Ericson and Larsson 1962, and Larsson 1966a). This is of importance in animal husbandry and many studies have been made to determine the effect of amino acid supplementation to foods used, for example in pig breeding (*cf* Clausen 1963, Tøllersrud 1961, and Larsson, Nilsson and Olsson 1966).

A low biological value of the food protein has a tendency to increase the fat content of the body (Clausen 1963, Munck 1964, Larsson *et al* 1966 and Larsson 1966a). It has therefore been suggested that the protein quality of the food is of certain importance for the etiology of obesity (Larsson *et al* 1966 and Larsson 1966a).

Of natural reasons it ought to be pointed out that supplementation of low quality protein with different protein "concentrates" has been practiced for many decades.

The present study was made to get more information as to the relation between the quantity and quality of the food protein and the development of nutritional obesity in different strains of mice.

### *Material and Methods*

Twenty two days old male mice of the Swiss, NMRI and CBA strains were used in the experiments. The various diets were given the animals *ad libitum* with free access to tap water. The mice were kept in macrolon cages 10×16 cm in size.

When the feeding period was ended all mice were killed by cervical dislocation. The carcass content of water, protein and fat was then determined as described in Chapter I.

TABLE VII

The composition of the three diets used in Chapter III Experiment I.

	Cereal food (A)	Group I (B)	Group II (C)
Dried skim milk			50.0
Fish meal		40.0	25.0
Sucrose	100	100	100
Dried brewer's yeast	50	50	50
L lysine HCl (feed grade quality)		38	15
DL methionine		13	05
Wheat meal	33	35.3	13

In all three groups the final food had the following composition

Concentrate (as listed above)	10
Oatmeal	15
Barley	30
Wheat meal	24
Wheat bran	21
	<hr/> 100 kg

*Experiment I* The composition of the three diets used in this experiment may be seen in Table VII. The vitamin and mineral supplementation of the diets was the same as in the standard laboratory food (Chapter I, Table I A). The content of digestible protein (in %) as judged by measurements after treatment with pepsin HCl was for the cereal diet 9.3, group I 11.6 and group II 12.4. The fat content of the three diets was (in %) 4.1, 4.2 and 3.9 respectively. The feeding period lasted for three weeks.

The weight of the mice was measured once a week.

TABLE VIII

The composition of the three diets used in Chapter III Experiment II

	Protein	Starch	Corn oil	Cellulose
Casein	100	550	50	300
Egg albumin	100	550	50	300
Wheat protein	100	550	50	300

*Experiment II* Also in this experiment three different diets were given the mice. The composition of the diets is given in Table VIII. The protein contents (in %) of the three diets were for "casein" 11.0, "egg albumin" 10.1 and for "cereals" 9.4.



## Results

Table IX indicates protein — fat ratio of the carcass after feeding the different diets for three weeks. It may be noted that the effect of the diets varies depending upon the strain of mice considered. Among the diets there is a considerable difference between diet A *vs* diets B and C. In general, diet C gives the highest protein — fat ratio, regardless of the strain.

TABLE IX

The protein fat ratio of the carcass in Swiss NMRI and CBA mice after feeding the three diets the composition of which is given in Table VII

Strain	Diet		
	A	B	C
SWISS	19	24	26
NMRI	18	23	25
CBA	10	18	21

The results from experiment II may be seen in Table X. The PER values indicate that in all strains there is a difference between the "wheat" diet and the two other diets (Fig. 7). The body fat determinations also found in table X indicate that in the Swiss mice no difference exist between the groups on the various diets. Figure 7 indicates however that the body fat contents of the NMRI and CBA mice vary due to the diet given.

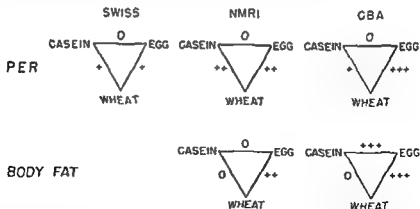


Fig. 7 Differences in PER (protein efficiency ratio) and in body fat content of Swiss NMRI and CBA mice given three isocaloric diets only varying in type of protein. Differences calculated after the values obtained in Table X according to Student's *t* test: +++ =  $p < 0.001$ , ++ =  $p < 0.01$ , + =  $p < 0.05$  and 0 = no difference.

TABLE X

The effect of the diets given in Table VIII on growth in g per g protein (PER) body fat content and protein fat ratio in Swiss NMRI and CBA mice Mean  $\pm$  s.e.m. 40 animals in each group Differences in Fig. 7

Diet	Growth in g per g protein consumed			Body fat content %			Protein — fat ratio		
	Swiss	NMRI	CBA	Swiss	NMRI	CBA	Swiss	NMRI	CBA
Casein	28 $\pm$ 0.15	29 $\pm$ 0.18	21 $\pm$ 0.16	101 $\pm$ 0.82	110 $\pm$ 0.83	130 $\pm$ 0.69	20	16	10
Egg albumin	28 $\pm$ 0.14	31 $\pm$ 0.19	25 $\pm$ 0.15	100 $\pm$ 0.77	95 $\pm$ 0.80	95 $\pm$ 0.72	22	23	21
Wheat protein	23 $\pm$ 0.18	20 $\pm$ 0.71	16 $\pm$ 0.17	115 $\pm$ 0.88	125 $\pm$ 0.72	140 $\pm$ 0.83	17	13	11

## Discussion

The results from this Chapter indicate that apart for the strain differences which again can be noted, the type of protein used in the various diets is of fundamental importance for the fat deposition in the body

It has been shown in several studies that when cereal products are used as basal food lysine is generally the first limiting amino acid with regard to the biological value of the protein when the interest is focused towards growth and protein efficiency (Ericson *et al* 1961, Ericson Larsson and Östholm 1962a, Clausen 1963 and Larsson 1966a) Also when consideration is taken into the effect of body fat deposition it is found that increased biological value of the protein in the food will decrease the body fat content (Ericson *et al* 1962b Clausen 1963, Munck 1964, Larsson 1966a and Larsson *et al* 1966) All these experiments were performed under strict conditions with regard to the food given The basal food also was of vegetable origin Usually the laboratory diets are based on cereals which are supplemented with "high quality" proteins, usually of animal origin This is also the case for foods used in animal husbandry During the preparation of food in particular lysine can be lost by different types of reactions Thus combination with other amino acids may give rise to unnatural peptide bonds involving the  $\epsilon$  amino group of lysine from which the lysine can be released by acid hydrolysis but not by digestive enzymes The  $\epsilon$  amino group of lysine can also be combined with aldehyde groups of sugars or other compounds from which it in some cases can be recovered after acid hydrolysis

In experiment I in this chapter two of the diets were supplemented with animal proteins The protein content of these diets could be regarded as comparatively low but of high quality On basis of earlier findings and analysis with regard to the content of lysine and methionine these amino acids were added (*cf* Ericson and Larsson 1962 and Larsson 1966a) The mice were only given the diets for three weeks which is enough to cause changes in the protein — fat ratio The Swiss and NMRI mice seem to react similarly to the diets while the CBA mice tend to be more sensitive to the improvement of diet A by the increased quantity and quality of the protein resulting in diets B and C In this experiment food consumption and utilization was not measured since the primary interest has been to study changes in the protein — fat ratio due to the strain and diet used In experiment II the three diets were isocaloric with protein contents as equal as possible Any change in the results thus was dependent on the quality of the proteins, within the particular

strain The feeding period was only two weeks, because it has been shown that the PER values of animal protein will decrease faster with time compared with cereal protein (Morrison and Campbell 1960) Further introductory experiments gave evidence for a faster decrease in PER in the NMRI mice than in the other strains Table X shows that the PER values between the casein and eggalbumin diets do not differ (Fig 7) On the other hand the wheat diet has lower PER than the two other foods It is evident that the explanation for this difference is due to a lack of lysine and partly of threonine since these two amino acids have been shown to limit the biological value of wheat (Ericson *et al* 1961 and Larsson 1966a) The feeding period was too short to expect the development of nutritional obesity to any extent On the other hand, if the body fat is plotted against the fat free body weight as described in Chapter I (Fig 1) one will find that the NMRI and in particular the CBA animals on the wheat diet will fall above the regression line In this connection it is of interest to note that the two experiments in this chapter seem to indicate that the CBA mice are very sensitive to the protein quality with regard to the parameters studied while the Swiss mice on the wheat diet have about the same PER as the CBA mice on the egg albumin diet Experiment II clearly indicates a qualitative difference between casein and egg albumin in the NMRI and CBA mice It has previously been shown that casein is inferior as protein food compared to egg albumin in the CBA mice (Munck 1964) The difference in biological value in this respect is most likely to be due to the relatively low content of cysteine and tryptophane in casein For the discussion on obesity which will follow in the other chapters it has to be pointed out that the Swiss mice on the diets used in these two experiments seem to be comparatively more resistant to changes in the protein quality



## CHAPTER IV

"Watch out for fads and fakes. There is perhaps no field so rife with charlatans as that of providing people with useless substitutes for long term maintenance of dietary discipline which is the key to obesity control."

*Sebrell 1957*

THE EFFECT OF A DIET COMPOSED OF THE AVERAGE 1960 CONSUMPTION IN SWEDEN COMPARED WITH A DIET SUGGESTED BY THE NATIONAL INSTITUTE OF PUBLIC HEALTH AND WITH A THIRD OF LOW CARBOHYDRATE AND HIGH FAT CONTENT, ON GROWTH BODY FAT CONTENT AND PROTEIN FAT RATIO IN TWO STRAINS OF MICE

by

STIG LARSSON

In a study by Blix *et al* (1965) a survey was made of the different nutrients consumed by Swedish people from 1925—1964, and the changes in consumption of these during this period. The results from this were related to suggestions about a revision of diet from a public health point of view. Among the changes in the consumption of different nutrients during these years, an increase of fat intake and a decrease in the use of bread and milk were noted.

In several studies it has been found that rats and mice are suitable animals on which to test different diets, particularly when the interest is paid to growth and fat deposition of the body (cf Ericson *et al* 1962*b* and Larsson 1966*a*). In such tests not only growth and food efficiency but also carcass composition with regard to fat, protein and water content are measured. The carcass composition is a function of dietary effects is of importance in problems concerned with obesity (Larsson 1966*a*) as well as with meat production in farm animals (cf Larsson *et al* 1966).

The present study was performed in order to compare the effect of "1960 years' diet in Sweden with a diet based on the suggested changes (Blix *et al* 1965) by measuring the parameters just mentioned. A third diet composed on the basis of suggestions by "food fadists" was also included in the study. This diet was poor in carbohydrate but high in fat content.

### *Material and Methods*

Twenty male mice of the Swiss and CBA strains were used in each group. The animals were 22 days old at the start of the experiment.

The main composition of the diets may be seen in Table XI. After mixing and grinding the various ingredients the mixture was run through a meat mill. The water content of the diets was decreased by transferring the food as thin layers upon baking pans. After this the pans were placed in an oven at 35°C with circulating air until the water content decreased to approximately 25%. The diets were then frozen until used.

The mice were given the diets *ad libitum* with free access to tap water. They were kept individually in macrolon cages (10×16 cm). The weight

of the animals was measured once a week, and the food consumption every day. The diets were never kept in the cages for more than 24 h without changing to fresh food.

After two weeks on the diets, the mice were killed by decapitation. Each animal was then subjected to carcass analysis as described in Chapter I.

TABLE XI

The composition of the three diets used in Chapter IV. A = 1960 diet; B = revised diet; and C = high fat, low carbohydrate diet.

	A	B	C
	g	g	g
Vegetables <sup>1)</sup>	2.8	4.6	4.0
Fruits and berries	12.5	15.4	13.4
Potatoes	17.1	19.1	2.0
Milk and cheese	29.5	29.6 <sup>2)</sup>	29.6
Meat, fish and egg <sup>3)</sup>	12.8	10.5 <sup>4)</sup>	35.5
Bread and other cereal products	14.6	22.3	0
Fat (butter, margarine and oils)	3.7	2.2	11.5
Theoretically calculated amount of calories per g food	1.8	1.6	2.1

Cellulose added to give 1.6 cal/g theoretically.

<sup>1)</sup> Green vegetables 65 %; dried peas and beans 35 %.

<sup>2)</sup> Sausages 20 %; ox meat 40 %; pork 20 %; cod 10 %; and whole eggs 10 %.

<sup>3)</sup> skim milk.

<sup>4)</sup> trimmed meat, no sausages.

## Results

Table XII shows the effect of the three different diets on growth, body fat content, protein — fat ratio and body water content in the Swiss and CBA mice. It may be seen that there is no difference in growth due to the various diets. The body fat content of the CBA mice is lowest in those given diet B, and highest in the animals given diet C (Table XII and Fig. 8). In the Swiss and CBA mice differences are noted between diet A and B with regard to body fat content. Both strains of mice given diet C have a significantly higher fat content than the other two groups (Table XII and Fig. 8).

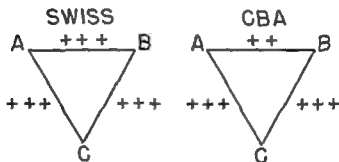


TABLE XII

The effect of the three different diets described in Table XI on growth body fat content protein fat ratio and body water content in male Swiss and CBA mice Mean  $\pm$  s.e.m. 20 animals in each group Differences in Fig. 8

Diet	Growth g in 14 days		Fat content % on dry wt		Protein — fat ratio		Water content %	
	SWISS	CBA	SWISS	CBA	SWISS	CBA	SWISS	CBA
A	10.5	8.7	28.2 $\pm$ 1.48	35.2 $\pm$ 1.91	1.9	1.6	68.3 $\pm$ 1.23	66.9 $\pm$ 1.02
B	11.1	8.5	21.3 $\pm$ 1.41	28.2 $\pm$ 1.81	2.6	2.0	69.2 $\pm$ 1.16	68.5 $\pm$ 1.11
C	10.8	8.9	36.3 $\pm$ 1.87	49.6 $\pm$ 2.51	1.4	1.0	61.4 $\pm$ 2.01	52.4 $\pm$ 2.13

### FAT CONTENT



### CARCASS WATER

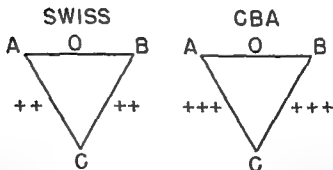


Fig. 8 Differences in body fat content and in carcass water of Swiss and CBA mice given three various diets A B and C, after values found in Table XII Differences calculated according to Student's *t* test +++ =  $p < 0.001$  ++ =  $p < 0.01$  and O = no difference

Table XII also shows that the protein — fat ratios largely follow the body fat content in the different groups. The body water contents of the mice from both strains given diet *A* and *B* do not reveal significant differences (Fig. 8). In both strains the water contents of those given diet *C*, however, have significantly decreased values compared with the other groups (Fig. 8). No differences in food intake between the animals on the three diets were noted.

### Discussion

In a recent study on the relation between the total caloric intake and the intake of single nutrients in Swedish food, Blix (1965) found that many groups in Sweden, particularly among women, did not eat sufficient amounts of calories. As a consequence of this finding it was realised that these groups did not consume enough protein. The study led to a survey on the total average consumption of various nutrients in Sweden (Blix *et al.* 1965) and ended with recommendations for a change in food habits. These changes were suggested on the basis of common public health opinion but no experimental studies were made. Among the recommendations a reduced fat intake was put forward and an increase in the consumption of skim milk, bread, potatoes and vegetables.

It has repeatedly been emphasized that the composition of the diet plays an important role for the etiology of obesity in various species (Larsson 1966a, Larsson *et al.* 1966 and Chapter III). It has to be pointed out that the diet composed on the suggestion of Blix *et al.* (1965) — diet *B* — gave the lowest body fat content in both strains. In the present study the different diets were offered the animals *ad libitum*. The strain differences in eating frequencies found in Chapter VII could therefore reflect the results. On the other hand it should be mentioned that the effect of the three diets in each strain revealed large differences especially with regard to body fat content and protein — fat ratio of the carcass.

The reason why the diet suggested by the Swedish National Institute of Public Health (diet *B*) gives more favourable values regarding low carcass content of fat and high protein — fat ratio than the diet composed of the average consumption in Sweden 1960 (diet *A*) seems to be different participation of fat. By decreasing the caloric portion of fat and sugar with corresponding increase in the calories coming from bread, milk (as

skim milk) and cheese and recommending trimming of the meat one obtains a diet where the biological value is relatively increased, without cutting down the necessary nutrients minerals and vitamins Diet C contained low amounts of carbohydrates including a decreased starch content due to a cut down in bread, cereals and potatoes The fat content of the diet was comparatively high but within physiological limits The motive for inclusion of diet C in the study is that semi scientific literature has from time to time contained advice such as "calories don't count" (Taller 1961) and related ideas such as in "the drinking man's diet" (Jameson and Williams 1964) Fenton and Dowling (1953) found that excessive carcass fat deposition was accelerated in some strains of mice by giving a diet high in fat content Fabry *et al* (1964) showed that rats fed *ad libitum* become fatter on a high fat diet than on a carbohydrate diet The present study also shows that diet C is without value if a decreased body fat content is desired When comparing the amounts eaten by the mice no significant differences were observed between the different groups In this connection it has to be pointed out that the three diets, at least theoretically, were isocaloric due to the dilution with cellulose Further, no essential differences in the growth rates were observed Nevertheless the amount of fat deposited in the body varied significantly depending on the diet given The protein content of the mice of the different groups was essentially the same Marked differences were observed when comparing the body content of water between diet A and B mice vs diet C mice where the water content was exceedingly low As has been shown by previous studies and in this publication, the water content of the body in obesity or in conditions with increased body fat is significantly lower than in other animals (Ericson *et al* 1962, and Larsson 1966a) Therefore it is clear that suggestions that a diet for obese individuals should contain high amounts of fat with little or no carbohydrate could be dangerous as obese subjects trying to loose weight according to such ideas would loose nothing but body water which is already in shortage In a study by Ericson *et al* (1962b) it was found that degenerative changes in the liver were very closely associated with low water content of this organ Further it was shown that the biological value of the diet protein was directly related to the fat content of the liver (Ericson *et al* 1962b and Larsson 1966a) In this respect there was also a direct relation between the fat content of the liver and that of the body (Larsson 1966a)

The mice given diet C especially from the CBA strain developed obesity — an obesity which could be regarded as nutritionally induced Even if the hereditary participation is obvious between the Swiss and

CBA mice receiving the same diet it is again of importance to stress that merely the type of diet is an etiological factor for obesity. Cohn *et al* (1965) have reported that force fed rats or those fed *ad libitum* deposited more fat when given a diet with high fat content free from carbohydrate which is in line with the results found in the present study in mice given diet C.

Most studies, however, have been undertaken with diets which could be considered as synthetical and highly concentrated with regard to the caloric content. In the present study even if the diets were dried due to the experimental conditions, cellulose was added to diet A and C. It is therefore clear that it was not only the caloric density of the diet that guided the nutritional fate of the mouse but the composition of the food given. Even if hereditary factors, also in this instance play a role one thus has to consider external factors such as nutritional in order to produce an obese mouse.

The results may indicate that with a relatively constant protein value other nutrients have the ability of causing increased fat deposition.

In the present experiments one has to take into account that the diets after mixing were dried at an elevated temperature. The biological value of proteins will decrease in diets stored or manufactured at higher temperatures particularly in the presence of reducing substances (*cf* Reinius 1955, Ericson and Larsson 1962 and Larsson 1966a). The primary cause for this loss is due to biological unavailability of lysine (Ericson and Larsson 1962 and Larsson 1966a). Although one has to consider a decrease in biological value of the diets used in this study, the temperature used when drying the food was only 35°C for a comparatively short period of time. Reinius (1955) made a diet composed to correspond with the average food of the Finnish people. He noticed that if the dried diet was kept at 140°C. for 30—40 minutes the result when giving this diet to rats was a complete cessation of growth presumably due to the lack of biologically available lysine. Under practical conditions in humans some of the diet constituents are unprocessed while others are fried or heated with water. Thus direct comparison between the results from the present study with humans cannot be made. On the other hand it might be assumed that the "strength" of the treatment of the food will vary from household to household. On the basis of the accumulated data on the effect of foodprocessing it is obvious that the biological value of various diets is probably less than that indicated in tables giving protein content and other data.



## CHAPTER V

"The simple observation reported herein may serve to demonstrate that seemingly minor portions of an experimental protocol may produce unrecognized effects

*Soyla 1966*

### PRODUCTION OF GOLDTHIOGLUCOSE OBESITY IN DIFFERENT STRAINS OF MICE

by

STIG LARSSON

In studying the LD<sub>50</sub> dose of goldthioglucose Brecher and Waxler (1949) found that a single intraperitoneal injection of the substance — 1 mg per g body weight — resulted in a high percentage of hyperphagia in the surviving mice finally leading to overweight and obesity. This observation initiated many studies on the nature and origin of this obesity. Thus Marshall Barnett and Mayer (1955) found that goldthioglucose produced specific lesions in the ventromedial parts of the hypothalamus that is the structures known to contain the satiety part of the feeding center (cf Brobeck 1955). Later studies have shown that goldthioglucose selectively accumulates in these parts of the brain (Liebelt and Perry 1957, Liebelt et al 1960, Debons et al 1962, Swartz, Christian and Andrews 1960 and Edelman et al 1965).

It has also been demonstrated that different strains of mice evidently do not have the same sensitivity to goldthioglucose with regard to the development of obesity (Liebelt et al 1960, and Larsson 1966b). In the course of the present study, goldthioglucose obesity was chosen for the following reasons. It was felt that this type of obesity was suitable for comparison with the nutritional obesity produced by certain foods. Further, the various strains of mice used in the present investigation have shown definite strain peculiarities and the preliminary study (Larsson 1966b) revealed these differences with regard to the reaction to goldthioglucose.

### *Material and Methods*

Male mice of the Swiss NMRI and CBA strains were used. They were fed *ad libitum* on the standard laboratory diet described in Chapter I and were given free access to tap water.

The mice were kept individually in the small sized (10×16 cm) macrolon cages.

Goldthioglucose as a 20 % oil suspension (Solganol B oleosum Schering) was administered intraperitoneally to the mice in the fed state or after 24 hours without food. The age of the animals when injected was 30—35 days. In connection with the injections of goldthioglucose

blood samples were taken and analyzed for glucose as described in Chapter I

The weight of the animals was measured once a week

## Results

Table XIII indicates that a dose of goldthiogluucose higher than 1 mg per g body weight given to CBA mice starved for 24 hours results in 100 % mortality, while the same amount given to starved Swiss and NMRI mice of the same age caused a mortality of only 10 %. The LD<sub>50</sub>-dose in these two strains was found to be about 2.0 mg per g body weight

TABLE XIII

Mortality of Swiss NMRI and CBA mice injected with different doses of goldthiogluucose S=mice starved for 24 h F=fed mice n=number of animals

Amount of goldthio glucose injected (m/g body wt)		Mortality in %			
		0.5 n=50	1.0 n=75	1.5 n=55	2.0 n=60
Strain					
Swiss	S	0	10	30	50
	F	20	90	100	
NMRI	S	0	10	20	50
	F	20	80	90	
CBA	S	40	80	100	
	F	100			

Table XIV A gives the incidence of obesity — calculated from the relation between the amount of body fat and the fat free body weight (Figure 1 Chapter I) — in surviving mice 60 days after the injection of goldthiogluucose. It may be noted that with injection of 1.0 mg per g body weight the incidence of obesity is highest in the CBA strain. Table XIV B indicates that the frequency of overweight follows the frequency of obesity only in the CBA mice whereas in the Swiss strain, overweight after the administration of goldthiogluucose is found only when the highest dose is given. In the NMRI mice a certain difference between the incidence of obesity and overweight is also noted.



TABLE XIV A

Frequency of obesity (see Fig 1) in % of surviving animals of the Swiss NMRI and CBA mice Starved animals

Strain	Dosage of goldthioglucose (mg/g body wt)			
	0.5	1.0	1.5	2.0
Swiss	5	30	40	50
NMRI	5	35	45	60
CBA	30	70		

TABLE XIV B

Frequency of mice with overweight (increased body weight compared with controls) in % surviving animals Starved animals

Strain	Dosage of goldthioglucose (mg/g body wt)			
	0.5	1.0	1.5	2.0
Swiss	0	0	0	15
NMRI	0	20	40	50
CBA	30	70		

The blood sugar values (in mg per 100 ml blood) in the three different strains after 24 hours starvation were in the Swiss mice  $47 \pm 2.5$ , in the NMRI  $82 \pm 5.8$  and in the CBA mice  $85 \pm 5.7$  (the difference between the Swiss mice and the two other strains being statistically significant  $p < 0.001$ ).

When goldthioglucose was injected into fed mice, a considerable increase in mortality was noted (Table XIII). On the other hand it was found that the incidence of obesity increased in the surviving animals.

### Discussion

In a preliminary study it was found that strain differences existed with regard to the development of obesity after a single injection of goldthioglucose (Larsson 1966b). Liebelt *et al.* (1960) showed that gain in body weight and mortality in mice following a single injection of goldthioglucose was dependent on the strain and the dosage. They also found a parallel response to the effects of goldthioglucose with regard to mor

ality and weight gain of CBA mice compared with the other strains studied. This was supported by the present study, and also shown to be the case with the final body fat content. It was also of interest to note that in general the data from the present study is in accordance with the findings of Liebelt *et al* (1960) suggesting that sublines of this inbred strain in another laboratory respond similarly.

The strain differences with regard to goldthioglucose in the present study related to those reported in the previous chapters seem to indicate that goldthioglucose is not only a suitable substance with which to produce obesity, but is also a substance which could be used for comparing different hereditary conditioned characteristics of other factors involved in causing obesity.

A 24 hour fast period preceded the injection of goldthioglucose in half of the animals under test. Determinations of blood sugar in these mice revealed that the Swiss variety had the lowest levels after the period of starvation (see also Larsson 1966b and Chapter II). It is possible that the resistance to goldthioglucose, both with regard to toxicity and the development of obesity, is partly due to the extreme fall in blood sugar in this strain. It has been reported that feeding the animals before the injection of goldthioglucose diminishes the incidence of permanent damage and hyperphagia by 30—50 per cent (*cf* Anliker and Mayer 1957) a result which could not be verified in the present investigation. Also in a study by Soyka (1966) it was found that the lethal effects of goldthioglucose were elevated in mice given glucose simultaneously with the injection of goldthioglucose. As the mortality increased, the incidence of obesity as judged from the body weight also increased. This finding, on the relation between the mortality and the incidence of obesity is confirmed by the present study. Soyka (1966) also showed that the administration of insulin decreased the mortality and the incidence of overweight when goldthioglucose was given. One explanation for these findings could be the blood sugar level *per se*. Edelman *et al* (1965) studied using various techniques, the accumulation of gold in the hypothalamus and the development of hyperphagia and obesity, in mice given goldthioglucose intravenously at different blood glucose concentrations. They found that the gain in body weight, the extent of the hypothalamic involvement and the degree of hypothalamic gold accumulation generally reflect the blood glucose concentration produced in the first few minutes following injection. The same authors presented a hypothesis correlating the blood glucose concentration with the degree of appetite inhibition. Hence the degree of appetite inhibition of the ventromedial area should be proportional

to the number of nerve cells activated. The generation of the action potential of the satiety neurones should also be dependent on the metabolism of a specific quantity of glucose in the glial cells. The hypothesis is confirmed by various biochemical and neurophysiological studies. Thus, differences in the incorporation of radioactive phosphorus into ATP and creatine-phosphate fractions as well as differences between the absolute amounts of these fractions in the hypothalamic areas in hungry and fed rats, emphasizes the specificity in the behaviour of the hypothalamic region (Forssberg and Larsson 1954 and 1955). Biochemical characteristics of the hypothalamus with regard to the fate of glucose have been found (Chain, Larsson and Pocchiari 1960), as have differences during hunger and satiety (Larsson 1967). The electrical activity of the hypothalamic region has also been found to be related to changes in the blood chemistry (Anand, Dua and Singh 1961 and Anand, Chhina and Singh 1962) and by the administration of amphetamine (Brobeck, Larsson and Reyes 1956).

In the present study, as previously mentioned, the incidence of obesity was lowest in the starved Swiss mice where the blood sugar values after a 24 hour fast were lowest. Preliminary studies where goldthioglucose was given to alloxan diabetic mice suggest, however, that this type of diabetic state with lack of insulin will contribute towards affording protection against goldthioglucose (Rerup and Larsson, unpublished). Thus it has been found that the injection of goldthioglucose in the alloxan diabetic mice will decrease the toxicity of this substance in spite of the exceptionally high levels of blood sugar. As mentioned by Soyka (1966) and further supported by the present study and by preliminary results obtained by Rerup and Larsson (to be published) it is a little surprising that the reaction towards a toxic substance can be altered by more or less physiological or endocrinological changes in the internal environment. Because of this goldthioglucose injected in smaller doses might prove to be a useful tool in differentiating between different types of obesity.

In line with the present opinion are the findings by Liebelt and Perry (1957) who noticed striking differences between two strains of mice regarding the susceptibility to goldthioglucose obesity. They explained this as being due to the varying sensitivity of the food intake — regulating mechanism to the toxic effect of goldthioglucose.

It is of interest to note the observations made by Oro, Wallenberg and Bolme (1965) on electrical stimulation of certain areas of the diencephalon and mesencephalon resulting in elevated levels of plasma FFA and glycerol both being indications of increased lipolytic activity.

Goldthioglucoſe injection reſults, among other things in an accumulation of gold in the diencephalon cauſing brain leſions (Swartz *et al* 1960) The areas damaged by the goldthioglucoſe are aſſociated with the regulation of food intake (Marshall *et al* 1955)

In the preſent ſtudy it was found that the development of overweight and obesity was aſſociated with a decreased tendency to mobilize fat In this connection it is of intereſt to note that the mice obese from the injection of goldthioglucoſe may have up to 100 times or more circulating insulin in the blood (Rerup and Larsson to be published) which may explain their decreased ability to mobilize fat This tendency was particularly ſtriking in the Swiss mice injected with goldthioglucoſe, which reſulted in an elevated fat content of the body, without overweight

With the accumulated data including previous and preſent reſults, it is tempting to ſuggeſt that the effect of goldthioglucoſe as a cauſe of obesity is dependent upon ſeveral factors It ſeems that the toxicity and the occurrence of obesity is increased in the fed ſtate (*cf* Edelman *et al* 1965 and the preſent ſtudy) The accumulation of gold in the ventromedial cells of the hypothalamus ſeems dependent on the glucose level of the blood As judged from the experiments by Edelman *et al* (1965) the accumulation of gold in the ventromedial nucleus is directly proportional to the blood glucose level, as well as to the percentage of overweight In the preſent ſtudy it was found that the incidence of obesity as judged from increased body fat content was higher in mice injected with goldthioglucoſe in the fed ſtate On the other hand mice made hyperglycaemic with alloxan injections ſeem to be leſs ſenſitive to the effects of goldthioglucoſe The injection of this ſubſtance in 24 h ſtarved alloxan-diabetic mice reſulted in a ſignificantly higher incidence of death than in the fed ſtate (Rerup and Larsson to be published) Therefore it could be ſuggeſted that the incidence of mortality depends on the levels of biologically active insulin Soyka (1966) found that hypoglycaemia cauſed by insulin decreased the mortality and the incidence of obesity in the goldthioglucoſe injected mice Earlier Drachman and Tepperman (1954) found that faſting prior to the injection of goldthioglucoſe increased the ſurvival rate in the mice Further it was found that adaption of the animals to a high fat diet prior to injection markedly enhanced the toxicity of goldthioglucoſe Whatever the explanation for the various reactions towards goldthioglucoſe may be, there is reaſon to ſuggeſt that the blood glucose level *per se* is not the primary cauſe



## CHAPTER VI

"A great deal of nonsense has been talked about this subject, and textbook after textbook has repeated the fallacy that almost unattainable levels of exercise are required to burn a pound of fat"

*Kennedy 1964*

### THE EFFECT OF MUSCULAR ACTIVITY ON FAT MOBILIZATION AND BODY FAT CONTENT IN NORMAL AND OBESE MICE

by

STIG LARSSON and SVEN JONSSON

Previous studies have demonstrated the importance of muscular exercise as a means of reducing weight in goldthiogluucose obese mice (cf Mayer 1955, and Larsson and Strom 1957). None of these studies, however, included body fat determinations. As has been pointed out earlier in this study, overweight and obesity are not necessarily the same condition and therefore the only safe method of studying factors that may influence obesity is to measure the body fat content. Parizkova and Strinkova (1964) found that in normal rats muscular exercise resulted in a lower fat deposition.

It has been shown in the preceding chapters that the mice exhibit strain characteristics towards some factors of importance for the etiology of obesity. It could therefore be possible that the effect of exercise should differ according to the strain used both in normal and obese mice. It was felt necessary that the exercise ought to be studied not only for its effects on the body weight but more important for its effects on the fat content of the body, food consumption and fat mobilization.

### *Material and Methods*

In this chapter only male mice have been used. Normal female rats and mice have an oestrus behaviour which is accompanied by an increase in spontaneous activity. Brobeck, Wheatland and Strominger (1947) have shown that the food intake is depressed during oestrus, leading to a weight loss while the opposite events take place during dioestrus.

Therefore in this investigation where other factors influencing food intake and fat deposition are studied it was felt that male mice were more suitable.

Swiss NMRI and CBA mice were used. They were fed *ad libitum* on the diet described in Chapter I and had free access to tap water.

Depending on the experimental conditions they were weighed once or twice a week.

After the experimental period the animals were killed. Part of the epididymal fat pad was usually removed and the net release of glycerol and FFA determined *in vitro* as described in Chapter I. The carcass content of fat, water and protein was determined according to Chapter I.

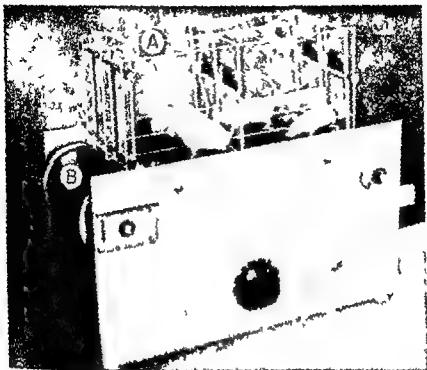


Fig 9 The arrangement for forced activity in the mice A=boxes where the animals are run on, B=running carpet

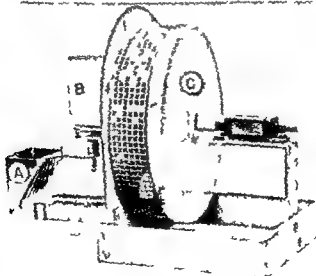


Fig 10 The arrangement for spontaneous activity in the mice A=feeding box B=water supply and C=rotating wheel



**Forced activity:** Mice of the Swiss, NMRI and CBA strains were subjected to a training regimen in a treadmill (Fig 9). The animals were gradually trained to run on the moving carpet at a speed of 25 cm/sec. The experimental period lasted for 30 days. The mice were running for 60 minutes in 6 consecutive 10 min periods where 2 min periods of rest were superimposed. When not in the treadmill the animals were kept individually in the small type of macrolon cages (10×16 cm) as did the controls. The food intake was measured daily.

Goldthiogluucose obesity was produced as described in Chapter I.

**Spontaneous activity:** The running activity of normal and goldthiogluucose obese mice of the Swiss, NMRI and CBA strains was measured. The apparatus may be seen in Figure 10. The animals were observed for periods of 30 days.

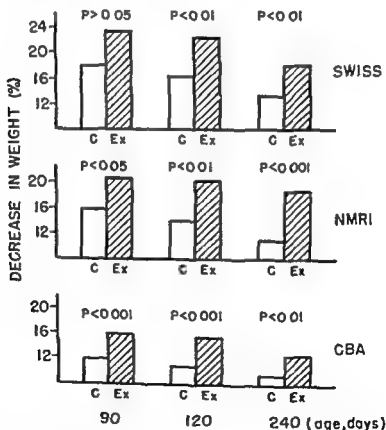


Fig 11 The effect of exercise in Swiss, NMRI and CBA mice on decrease in body weight after 4 h of fast compared to non exercised fasted animals. The decrease in weight in %.

*Influence of cage size* Normal mice of the Swiss, NMRI and CBA mice were kept individually in either the smallest sized cages (10×16 cm) or in the largest sized (23×39 cm) for 60 days after weaning after which the carcass analysis was performed. All animals were given the standard laboratory diet *ad libitum*.

Groups of exercised mice were starved for 24 h and the decrease in weight recorded.

## Results

*Forced activity* Table XV shows the effect of forced exercise in normal mice of the Swiss, NMRI and CBA strains. It may be seen that the NMRI mice did not change in weight on forced activity compared with the controls, while both the Swiss and CBA mice had a negative weight change. The same table also indicates that the Swiss mice had the same food intake over the 30 day period whether they were forced to run or not. Both the exercised NMRI and CBA mice had significantly increased food intakes compared with the unexercised controls. After the period of forced activity the NMRI and CBA animals had significantly lower fat contents. The muscular activity caused an increase in the protein-fat ratio in all the strains studied.

As shown in Table XVI forced activity in all the three strains resulted in higher net release of glycerol and FFA from the adipose tissue *in vitro*. The response to adrenalin *in vitro* did not change from the controls.

TABLE XV

The effect of forced activity (1 h per day for 30 days) in normal male Swiss, NMRI and CBA mice. Age of the animals at the start of the experiment was 90 days. Ten animals used in each group. The changes are related to the values in unexercised controls. Differences calculated according to Student's *t* test. a) =  $p < 0.001$  b) =  $p < 0.01$ .

Strain	Weight change g	Change in food intake g	Change in body fat content %	Change in protein/ fat ratio %
Swiss	-5.9 <sup>a</sup>	-4	-15	+3.3 <sup>a</sup>
NMRI	-0.6	+55	-23 <sup>b</sup>	+7.9 <sup>a</sup>
CBA	-2.0 <sup>b</sup>	+43	-30 <sup>b</sup>	+8.1 <sup>a</sup>

TABLE XVI

The change in net release of glycerol and FFA from adipose tissue *in vitro* after forced activity in Swiss NMRI and CBA mice. Differences calculated according to Student's t test a) =  $p < 0.001$  b) =  $p < 0.01$

Strain	Change in net release from adipose tissue in % of values of controls	
	Glycerol	FFA
Swiss	+35 <sup>b)</sup>	+36 <sup>1</sup>
NMRI	+46 <sup>1</sup>	+51 <sup>1</sup>
CBA	+6 <sup>1</sup>	+62 <sup>1</sup>

*Spontaneous activity* Table XVII shows the effect of spontaneous activity followed to 30 days in obese male mice (from 30 days of age) of the Swiss NMRI and CBA strains. It may be noted that the weight gain of the "controls" is more than twice that of exercised mice in general. The same Table shows that the fat content of the body in all strains after activity is significantly lower than in the controls and that in obese animals the carcass fat content has gone down to the value of normal animals. The protein — fat ratio in these animals also correspond to that of the non obese mice.

TABLE XVII

The effect of spontaneous activity in squirrel wheels on weight and body fat content in obese Swiss NMRI and CBA mice compared with unexercised animals. Differences calculated according to Student's t test a) =  $p < 0.001$

Strain	Weight change %	Change in body fat content %
Swiss	-80 <sup>1</sup>	-42 <sup>1</sup>
NMRI	-55 <sup>1</sup>	-68 <sup>1</sup>
CBA	-82 <sup>1</sup>	-85 <sup>1</sup>

*Influence of the cage size* Table XVIII shows that the animals kept in the small cages have a significantly higher body fat content.

Figure 11 shows that exercised animals decrease more in weight on starvation than unexercised controls.

TABLE XVIII

The effect of the cage size in normal Swiss NMRI and CBA mice on the body fat content. Mean  $\pm$  s.e.m. 30 animals in each group. Differences calculated according to Student's t test a) =  $p < 0.001$  b) =  $p < 0.01$

	NMRI	Swiss	CBA
Large cage	26.3 $\pm$ 1.33 <sup>b)</sup>	21.4 $\pm$ 1.73 <sup>1</sup>	30.7 $\pm$ 1.42 <sup>1</sup>
Small cage	31.3 $\pm$ 1.00	29.5 $\pm$ 1.34	36.4 $\pm$ 1.27

## Discussion

The results from this study indicate that the muscular exercise will decrease the fat content of the body with or without a weight change of the body

The effectiveness of exercise in the treatment of obesity has been discussed earlier (cf Mayer 1955) but surprisingly little practical application has been made. This depends no doubt upon the experimental set up. If experimental animals are exercised or human beings subjected to muscular activity, observations have been made that the body weight often shows little or no decrease. This has led to the conclusion that exercise is of minor importance in the treatment of obesity. This could of course be true under certain circumstances if one considers obesity and body weight as primary dependent upon each other. As has been discussed elsewhere in this study, obesity is not always followed by an increased body weight but should rather be recognized when the fat content of the body is higher than normal.

Judging from the results on the effect of exercise in mice where the animals are starved for 24 hours (Fig 11) it is evident, that all animals, regardless of strain and age will decrease more in weight than corresponding non exercised mice without food. This simple finding suggests that the exercised animals have a higher ability to mobilize fat. In view of the findings from the *in vitro* studies this is not surprising since the fat mobilization as measured by the net release of glycerol and FFA is increased in the exercised animals. Mayer (1955) has pointed out that exercise entail a considerable expenditure of energy. It is difficult to understand why muscular exercise has received such a negligible attention in many studies where the interest has been focused on the causes and treatment of obesity. One of the arguments used, is that muscular activity requires little caloric expenditure when performed moderately. An other argument is that physical activity is accompanied by an increased food intake which would cover the extra calories utilized. Still, as pointed out by Parizkova (1966) the increased energy output due to enhanced physical activity in sportmen may require correspondingly increased caloric input reaching 7000—8000 kcal per day. In these people the high intake of calories will lead to a high ratio of lean body mass at the expense of fat. Miller and Mumford (1966) have discussed the metabolic situation in humans subjected to moderate exercise. They give evidence for the view that the extra energy output *per se* during exercise may not be the important factor as pointed out by Mayer (1955) but rather the *last*

increase in metabolic rate following upon muscular activity. Exercise or electrical stimulation of the skeletal muscles results in increased fatty acid utilization in muscles (George 1964, and George and Vallyathan 1964). Further, as shown by Parizkova (1966) the amount of FFA released *in vitro* from the epididymal adipose tissue is higher in running animals an observation which can be confirmed by the present study. Increased sensitivity to adrenaline with regard to the release of FFA has also been noted after exercise (Parizkova 1966).

From a physiological point of view the present results seem to indicate that exercise is of fundamental importance in relation to the fat content of the body as well as to the fat mobilizing capacity. In this connection Oro *et al* (1965) have given indirect support in studies on the effect of diencephalic and mesencephalic stimulation in anaesthetized dogs on the release of FFA and glycerol. In these brain parts the localization of functions related to locomotor activity as well as autonomic functions such as increased heart rate, blood pressure and muscular blood flow are found (Rushmer *et al* 1961). Studies in experimental animals suggest a close integrative relation between the central areas regulating food intake and locomotor activity (cf Kennedy 1964). Thus when lesions are produced in the ventromedial parts of the hypothalamus the resulting hyperphagia is always accompanied by decreased spontaneous activity.

It can be discussed whether the "normal" laboratory conditions for mice and for other experimental animals are physiological in a true sense. The animals are usually kept in comparatively small cages with little opportunity to move freely. Normally all animals exhibit a certain degree of spontaneous locomotor activity. When this opportunity is lost or reduced it has been shown that the immobilization may lead to obesity (Ingle 1949). Mayer *et al* (1954) have suggested that a non responsive range exists with regard to change in food intake on both sides of the usual zone of activity. The present experiments confirm these findings by demonstrating that depending upon the size of the cage the fat content of the body will vary regardless of the strain of mice used.

Tepperman and Tepperman (1964) have introduced the term adaptive hyperlipogenesis from a practical point of view. The same authors have later applied and extended this term and found it be valid for various forms of experimental obesity (cf Tepperman and Tepperman 1965). Without taking into the discussion the adequate intermediate stimulus it seems logical to include muscular inactivity into the term adaptive hyperlipogenesis since inactivity, as found in the present study always

leads to increased body fat deposition and a decreased ability to mobilize fat in the fed state as well as after various periods of food deprivation.

The energy cost of exercise is approximately proportional to body weight. From this follows that an overweight individual will require more energy for the same amount of work than would a lean individual. In the present study forced activity in obese mice proved to be effective not only with regard to weight reduction but in particular to decrease the body fat content. This was also true for non-obese exercised animals.

Hypothalamic obesity in the rat and mouse is characterized by a considerable degree of inactivity (cf. Kennedy 1964 and Mayer *et al.* 1954). This evidently is depending on the extent of the hypothalamic lesion as it seems as the same areas of the hypothalamic region is also governing the locomotor activity (cf. Kennedy 1964). The obesity caused by goldthio-glucose only partly follows the pattern of hypothalamic obesity. As previously shown and confirmed by the present experiments the goldthio-glucose obese mice are primarily inactive when placed in a squirrel wheel (Mayer 1964 and Larsson 1957). As demonstrated previously and confirmed by the present study the obese mice gradually increase the spontaneous activity so that they loose weight and body fat. Mayer (1955) found that the spontaneous activity in goldthiogluucose obese mice is not impaired. Mayer *et al.* (1954) have suggested that within the so called sedentary range the hunger-satiety mechanism is not working properly. Thus within this range the food intake of animals is partly exceeding the actual caloric needs. By exercise the sedentary range is avoided which in normal individuals will cause the regulatory mechanism to act more accurately. An example of this found in this chapter, is the observation that the size of the cage is of importance for the extent of fat deposition of the body. It has also been reported that exercise up to about 6 hours per day in rats will result in increased food intake but no weight change (cf. Mayer 1955). In the present study no attempts were made with longer periods of forced exercise than one hour. The CBA and NMRI mice subjected to this mild type of exercise responded with a significant increase in food intake. In spite of the increased food intake the CBA mice lost weight while no weight change was observed in the NMRI mice. The Swiss mice subjected to forced activity did not compensate the extra caloric expenditure with increase in food intake. As a natural result of this the animals lost weight. However in all the animals the forced activity decreased the body fat content. In the CBA mice roughly 50% of the total body fat disappeared after 1 hour of forced activity daily for 30 days. Still in this strain the increase in food intake was not

was about 40 % more than the unexercised controls kept in small cages. The relative weight loss, however, was only about 10 % compared to the controls. It thus seems justified to state that under the experimental conditions described, exercise is an effective factor for weight control and which is more important, as a regulator for the fat content of the body. It has also to be pointed out that strain differences exist, but the general trend is not changed by this fact.

The present chapter shows that when studying problems concerned with body fat content, lipogenesis and lipid mobilization in relation to obesity, the simple determinations of body fat weight and food intake are enough to get information of the net effects on the organism. To some extent, however, it is of interest to study some of the "local" parameters for example in the adipose tissue. Friedberg *et al* (1960) have found that acute exercise lowers the plasma FFA content followed by a significant rise in plasma FFA concentration immediately after exercise. The rise in FFA concentration after exercise indirectly points towards an increased mobilization of fat. Further, Fritz *et al* (1958) studied the fatty acid oxidation by skeletal muscle during rest and activity *in vitro* and found that stimulated muscle utilized twice as much fatty acids as corresponding muscle at rest. The present study indicates that the exercised animals have a higher net release of glycerol and FFA *in vitro* from adipose tissue. Preliminary experiments seem to indicate that the muscles from exercised mice utilize more fatty acids than the unexercised muscle even without stimulation. All experiments in the same line seem to indicate that exercise not only will facilitate the adipose tissue to a higher fat mobilizing capacity but also facilitate the muscle to utilize more fatty acids even at rest.

Regarding the animals living in small cages with little opportunity to exercise spontaneously it is evident that the sedentary range is a border line in nutritional homeostasis which deserves much attention. Thus, it can be stated that muscular inactivity is an important factor for the etiology of obesity.

## CHAPTER VII

"Nearly all of the published studies of the regulation of food intake neglect the fact that the total amount of food eaten is always the product of two factors, the number of meals multiplied by the intake of the average meal

*Brobeck 1955*

### EATING BEHAVIOUR IN NORMAL AND OBESE SWISS NMRI AND CBA MICE

by

STIG LARSSON



It has been demonstrated in several investigations that the consumption of the daily food ration within short periods considerably increases lipogenesis in rats (Tepperman *et al* 1943, and Hollifield and Parson 1962) Attempts have been made to repeat these experiments in mice, but without success probably due to the higher metabolic rate in these animals which causes difficulties in maintaining the body weight by too infrequent feedings (Larsson, unpublished) In a previous study it was shown that goldthioglucoase obese ASw mice ate larger meals less frequently than non obese controls (Larsson and Strom 1957)

In view of other strain differences found in this study, it was of interest to measure the eating frequency in the three types of mice used in this investigation, both in lean and obese animals

### *Material and Methods*

Male mice of the Swiss, NMRI and CBA strains were used The age of the animals at the start of the experiment was 150 days Goldthioglucoase obesity was produced as described elsewhere (Chapter I) and the compound injected when the mice were 45 days old At the start of the experiment the obese mice had reached the static phase of obesity

To record the meal frequency the same apparatus as previously described was utilized (Larsson and Strom 1957) The observation time for each mouse in the apparatus was about 14 days The food used was the regular standard food described in Chapter I, formed into small tablets fitting in the feeding apparatus Because of technical reasons about 15 % adhesive material in form of *sacch lactis* was added

### *Results*

Table XIX and figure 12 indicate that normal Swiss mice ate more frequently than did the NMRI mice which in turn had a higher meal frequency than the CBA mice The goldthioglucoase obese mice all ate less

frequently than the corresponding lean litter mates (Table XIX). The obese NMRI and CBA animals ate significantly less frequently than the obese Swiss mice.

Figure 12 shows the differences between the various strains indicated by the same system as previously used by Munkner (1963) for other parameters. As the food intake did not differ significantly between the lean and fat mice the size of each meal in the obese animals was much larger than in the non obese litter mates.

TABLE XIX

Eating frequency in 24 h in normal and goldthioglucose obese male Swiss NMRI and CBA mice studied for two weeks in the feeding apparatus. Differences between lean and obese mice from the same strain calculated according to Student's t test. Number of animals in each strain: 15 normal and 10 obese. a)  $p < 0.001$ .

Strain	Eating frequency in 24 h	
	Normal	Obese
Swiss	$39 \pm 1.0^a$	$18 \pm 0.8$
NMRI	$27 \pm 0.7^a$	$12 \pm 0.8$
CBA	$19 \pm 0.8^a$	$10 \pm 1.1$

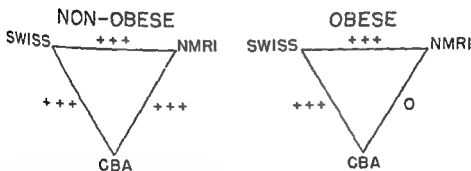


Fig. 12 Difference in eating frequency between normal and obese Swiss NMRI and CBA mice, calculated after the values given in Table XIX according to Student's t test.  $+++ = p < 0.001$  and  $0 =$  no difference.

### Discussion

When studying the regulation of food intake and the factors of importance for the etiology of obesity such as various nutritional strain characteristics in normal and obese mice it is obviously important not only to

measure the total food consumption but also to record the eating behaviour in the animals over an extended period of time

In a previous study it was revealed that goldthiogluucose obese mice of the ASw strain ate larger meals of food less frequently compared with their corresponding lean litter mates (Larsson and Strom 1957). In the present study this observation was confirmed in all three strains. It was however also shown that particular strain characteristics existed with regard to the feeding frequency. Thus, the Swiss mice, exhibiting many indications of "lean" mice, ate significantly more frequently than the other strains. It is possible that this phenomenon, as such contributes to the lower body fat content generally found in this strain compared with the NMRI and CBA mice. It has been demonstrated that rats can be trained to eat sufficient amounts of food in one hour per day, and studies of the respiratory quotients of these animals indicate increased lipogenesis (Tepperman *et al* 1943). Hollifield and Parson (1962) have later shown that adaptive changes occur in adipose tissue in rats fed for only two hours per day. Thus in these animals the FFA content fell as the rate of lipogenesis increased. Other studies have further revealed that the total body metabolism is markedly altered by various eating habits such as changes in eating frequencies "meal eating" vs nibbling (Cohn and Joseph 1960). Similar studies have suggested that less frequent eating but larger meals might lead to obesity. Van Putten, van Bekkum and Querido (1955) found that when rats with hypothalamic hyperphagia were pair fed against control animals, the hyperphagic animals ate the daily ration in a short time but the control rats needed twenty four hours to eat their diet. The hyperphagic rats contained about twice the amount of fat but less protein than the control rats. The present results are largely in the same line as those quoted above as it seems that those mice eating less frequently also have the highest body fat content.

Further evidence for the importance of the eating frequency for fat deposition is the finding by Feldman *et al* (1957) that male chickens made aphagic with diencephalic lesions and therefore tube fed developed grossly increased quantities of subcutaneous fat.

The present experiments again indicate the importance of taking into consideration the strain characteristics when studying nutritional problems. From a practical point of view, hereditary factors in obesity must be considered. In clinical studies this has not always been the case. Rather existing genetic basic characteristics are often regarded as psychological factors because of lack of compiling data from experimental animals with clinical conditions in humans.

In the literature very few studies are found concerned with the importance of eating frequencies for the final body composition. Anliker and Mayer (1957) found certain differences between the feeding behaviour in various types of obesity. They used the Skinner box to observe the meal frequency. This apparatus works so that the animal has to press a bar in order to receive the food possibly in reward. In the present study, as has been explained previously (Larsson and Strom 1957), the food is available in the cage the whole time which avoids psychological interference as to the question whether the animals have to work for the food or not.

In the present study the constancy of the meal frequency within the respective strains is surprising and might be taken as evidence for the strength of the genetic impact on the nutritional state. Then, as shown in Chapter I, III, IV and VI, external factors such as food composition and muscular activity are of importance in altering and modifying the hereditary destiny.

As changes in the dietary pattern can induce significant metabolic alterations (*cf.* Tepperman and Tepperman 1965) it is not surprising that studies in meal fed and nibbling rats reveal that the metabolism of the adipose tissue in the meal eating animals is characterized by a marked increase in those metabolic parameters leading to the synthesis and deposition of carbohydrate and fat (Leveille and Hanson 1965). The obese mice of all the strains studied have significantly lower feeding frequency than their non obese litter mates. As the obese mice used had reached the static phase of obesity, i.e. not gaining further weight or body fat content, it is possible that the meal frequency is of importance to pattern the machinery of the fat cell in the obese animals towards enhanced lipogenesis or to maintain it at this state. There is good evidence for such an adaptation in meal eating animals (Cohn and Joseph 1959 and Leveille and Hanson 1965). It is of course impossible to state whether the differences in the eating behaviour are the cause of the increased body fat content in the mice eating less frequently or if this phenomenon runs parallel to the high body fat content.



## CHAPTER VIII

"Most dietary experiments imply the assumption that the animal consumes as much of the available food mixture as will improve the animal's nutritive status. Trust is placed in paired feeding as the sole control procedure

*Adolph 1947*

### FOOD PREFERENCES IN DIFFERENT STRAINS OF NORMAL AND OBESE MICE

by

STIG LARSSON

In a previous study, it was found that mice of the ASw strain, made obese with goldthioglucose usually gained weight when the food was changed from the ordinary laboratory diet to a free selection of three different diets (Larsson 1957). While gaining weight the mice in general preferred to select a diet with a high fat content. The normal mice did not change significantly in weight on free selection.

It has been found that the qualitative content of the food is of importance for the development of obesity (Lundbaek and Stevenson 1947 and Strominger, Brobeck and Cort 1953). The present results also provide evidence for this observation.

Fenton and Dowling (1953) have produced evidence for the view that different strains of mice react differently with respect to body weight when offered a diet with a high fat content. The present investigation has also revealed that both hereditary and nutritional factors are important for changes in body composition which may lead to obesity.

The present experiments deal with the free selecting of diets by the three strains of mice used throughout this investigation in order to explore whether differences exist in the preferences of mice for the various diets. To make this study more complete free selection in experimentally induced obesity (with goldthioglucose) was also measured. Besides for the food preference and growth determinations the carcass content of water, protein and fat was also measured.

### *Material and Methods*

Male mice of the Swiss NMRI and CBA strains were used in this study. All mice were fed *ad libitum* with free access to tap water. They were given the standard food described in Chapter I until 120 days of age when they were given a free choice of three different diets. Table XX gives the composition of the diets. The mice on free selection were kept individually in macrolon cages (20 × 26 cm) and the different diets given as may be seen in Figure 13. The corresponding controls were placed in the same sized cages and received the standard diet throughout their lives. Goldthioglucose obesity was produced as indicated in Chapter I. The

obese animals were not used until they reached the static phase of obesity. This is the reason why all animals were fairly old (120 days) when used in the actual experiment.

TABLE XV

The composition of the three diets used in the free selection study

Content in g	A	Diet B	C
Egg albumin	250	700	220
Sugar	680	230	220
Corn oil	30	30	60
Lard			460
Premix A )	20	20	20
Premix B*)	20	20	20

\*) Premix A had the following composition and contained per g: vit. B 0.25 mg, nicotinamide 1.00 mg, folic acid 0.01 mg, calcium pantothenate 1.00 mg, vit. A 2.10 mg (325 IU/mg), vit. D 0.225 mg (300 IU/mg), vit. E 1.25 mg and brewer's yeast up to 1 g.

Premix B contained per g: sodium chloride 200 mg, brewer's yeast 300 mg, dicalcium phosphate 500 mg, cobalt sulphate 2.5 µg, manganese sulphate 0.5 µg, ferrosulphate 1.65 mg, choline bitartrate 200 mg and magnesium sulphate 500 mg.

The food intake was measured daily and the body weight once a week. As a certain incidence of diabetes was observed in the obese mice in a previous study on free selection (Larsson 1957) the urine was frequently checked with "Clinistix" (Ames) for the occurrence of glucosuria.

After 30 days on free selection the animals were killed and the carcass content of water, protein and fat was measured as described in Chapter I.

## Results

As may be seen from Figure 14 there is a difference in the selection of diets in the non-obese mice depending upon the strain. The NMRI mice seem to prefer a diet lower in fat content than the Swiss and CBA mice. The non-obese Swiss and CBA mice select a diet which in average contains more than 50 caloric % of fat.

The obese mice, regardless of the strain, have a higher fat intake than their respective non-obese litter mates (Fig. 14). Between the obese mice



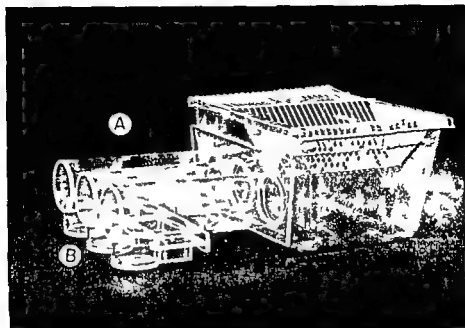


Fig 13 The arrangement used for the free selection studies A=funnels leading to the food cups=B

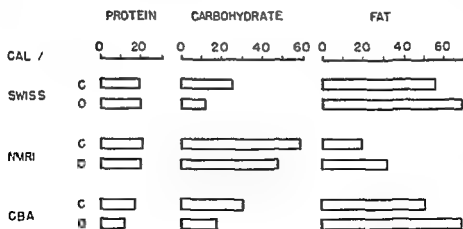


Fig 14 The selection of food in normal (c) and obese (o) Swiss NMRI and CBA mice expressed as calories. participation of protein carbohydrate and fat in %

of the various strains the selection of food is different, the obese Swiss and CBA mice having the highest fat intake. The obese NMRI mice gradually decrease the fat intake with corresponding increase in the consumption of carbohydrate (Fig 15). In the obese CBA mice with a high fat intake of about 70 caloric % no decrease with time was noted (Fig 15). Obese Swiss mice select a diet with high fat content for a period of roughly 18 days after which the fat consumption decreases.

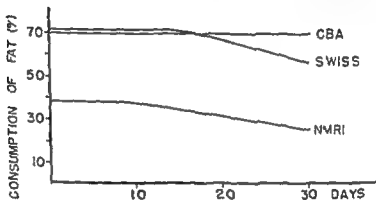


Fig 15 The caloric participation of fat in the food in % in obese Swiss NMRI and CBA mice in relation to time

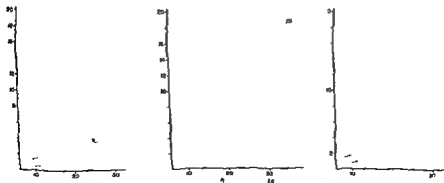


Fig 16 The body fat plotted against the fat free weight of Swiss NMRI and CBA mice on 30 days of free selection of food X=normal mice on free selection ●=obese mice on free selection and O=obese mice on the standard laboratory diet. As a comparison the broken lines indicate the limit values for normal mice on the standard laboratory food as given in Fig. 1

Figure 16 shows that the body fat content of the animals on free selection always was higher than in the corresponding mice on the standard laboratory diet. It may be seen that the difference is particularly large in the NMRI and CBA mice. As a consequence of the increased body fat content the protein — fat ratio in the carcass will decrease.

## Discussion

Largely the results obtained earlier on free selection of diets in obese ASW mice have been confirmed (Larsson 1957). All obese mice consumed significantly more fat than corresponding lean litter mates. The obese Swiss and CBA mice had the highest relative fat intake. This is in line with the other observations in the CBA strain obtained in the present publication. Thus the CBA mice have a comparatively high body fat content on the standard laboratory diet and a comparatively low release of glycerol and FFA from the adipose tissue *in vitro*. This strain has also been found to be very sensitive to the composition of the diet and the quality of the protein in the food with regard to the fat deposition in the body. The Swiss mice on the contrary being of the "lean" type have largely the same selection of calories, suggesting that in this strain other mechanisms might be governing the body composition.

The obese mice become fatter after the period on free selection but with regard to the NMRI and CBA strains the weight was unchanged compared to the previous period on the standard laboratory food. The obese Swiss mice however invariably became fatter *with* overweight compared with the preselection period. The non obese mice on free selection did not change in weight compared with the non obese animals on the standard food.

It is of certain interest to note that even if the selection of food among the different strains varied all animals turned fatter. The standard diet as shown in Chapter I promotes growth and has a comparatively high feed efficiency regarding the biological value of the protein. In addition this food results in a comparatively low fat content of the body and high protein — fat ratio in the normal mice. The protein present in the diets used in the food preference study, egg albumin, is considered to be a well balanced protein regarding the amino acid composition (Munck 1964, FAO 1965 and Chapter III). The increased content of fat in the carcasses of normal mice on free selection obviously must be caused by the fat or

carbohydrate in the diets. In addition one has to bear in mind that the caloric density of the three "synthetical" diets was higher than in the standard food. The protein was represented by egg albumin in all three diets since it was shown that obese ASw mice without protein would select a diet almost without protein (Larsson 1957).

It could naturally be argued that this type of experiments does not reveal anything but a mixture of gustatory and other phenomenon. Against such objections, Richter and Barelare (1938) have concluded "The amazing consistency of the choices made by the animals indicates that the changes do not depend on a trial and error process. It would seem far more likely that they are due to an altered chemistry of the taste mechanism which reflects the altered chemistry of the rest of the body during pregnancy and lactation." Richter and Barelare (1938) thus suggested that a normal animal would select the best choice of not only common nutrients but also vitamins and minerals. In part this was confirmed by Larsson (1957) but still the strain differences found in the present study remains. In relation to the results by Richter and Barelare (1938) it is evident that beneficial choices exist during pregnancy and lactation (Larsson 1957). In this case increased nutritional demands naturally exist. The present study indicates however that nutritional obesity can be produced with a choice of either high carbohydrate or high fat intakes depending upon the strain used.

The significant differences in the selection of diets in normal mice depending on the strain are of interest since it proves that results obtained in one laboratory not necessarily have to be identical with results obtained in other places due to differences in diet composition or the strains used. Such differences are likely to exist also in humans. Therefore when studying problems concerning nutrition one should avoid categorical statements to be true for all individuals without investigating the external environment in relation to the internal, especially when studying prophylaxis or treatment of obesity.

The differences in body fat contents of normal mice subjected to free selection compared with litter mates on the standard laboratory diet are particularly noticeable in the NMRI and CBA mice. These two strains in particular on free selection developed what could be called nutritional obesity. This is partly also true for the Swiss mice. It has to be noted that neither of the strains had a significant increase in body weight compared with the controls on the standard diet. In the previous study on free selection it was observed that the obese ASw mice often gained weight for a short period of time to reach a higher weight level (Larsson 1957). During

this period of weight gain, the mice selected a diet extremely high in fat content. A similar observation was noted for the obese Swiss and NMRI mice. These animals selected in the beginning a diet which was high in fat but gradually decreased this intake. The only strain of obese mice that had an appreciable weight increase on the free selection was the Swiss strain. It has been shown previously that the Swiss mice are fairly resistant to nutritional obesity. In these studies the protein quantity and quality have been kept under consideration. On free selection, however, it is obvious that even this strain will develop what could be called nutritional obesity. When the new weight level was reached the obese mice gradually decreased the intake of fat and finally selected a diet with equal caloric participation of carbohydrate and fat (Larsson 1957). In this study as mentioned above this was also found in the present chapter. It was also found that the obese CBA and Swiss mice on free selection continuously preferred a diet with high fat content. On the contrary the NMRI mice selected a diet with a comparatively low fat content. Still they become obese.

In the literature comparatively few studies have been concerned with free selection studies in normal and obese animals. Lundback and Stevenson (1947) found a reduced carbohydrate intake after fat feeding in normal rats and in rats with hypothalamic hyperphagia. Mayer (cf. 1955) studying the hereditary hyperglycemic obese mice found that these animals developed obesity in diets with high content of carbohydrates. Free selection was not performed in these experiments. Michelsen, Takahashi and Craig (1955) and Barboriak *et al* (1958) described the development of obesity in rats fed for a long time a high fat diet. Masek and Fabry (1959) could not support these findings when rats were given isocaloric high fat, high carbohydrate or high protein diets. It has to be pointed out, however, that the animals referred to above were not on free selection but were offered one of the diets and then compared with other animals offered an other food.

In part this explains the discrepancies as being due to the higher caloric density of the high fat diets usually applied. In *ad libitum* fed mice as described by Chapter IV the animals regardless of the strain will increase the body fat content on a high fat diet.

If an animal consuming carbohydrates instead of fat will develop significant increase in body fat content it is obvious that the part of the metabolism going through the carbohydrate-lipid metabolic cycle is high. The experiments suggest that this might be the case for the NMRI mice where a comparatively low fat consumption still can produce body fat con-

tents of 50 %, calculated on dry weight, even in "normal animals. In a study by Fabry *et al* (1964) it was suggested that when the effect of the pattern of food intake on the carcass composition in animals receiving diets with varying fat content was followed one probably has to take into consideration "differences in diet composition and its application, duration of feeding, rat strain used *etc*. This view is entirely agreed with in the present investigation. Further, previous results have revealed that obese A5w mice eat less frequently but in larger meals compared to lean litter mates. The present study has also demonstrated strain differences with regard to eating frequencies and to the size of the meals eaten. Thus the spontaneous eating frequencies were lower in the CBA and NMRI mice when compared with the Swiss animals. In this experiment the standard diet was given to groups of the animals. These had in every single animal a lower fat content of the body than the corresponding control.

In summary these studies show that the strain differences are powerful in determining the selection of food. Still, with a free choice of food all three strains will increase the fat content of the body in a degree which would transfer previously normal mice to the category of obese individuals.



## GENERAL CONCLUSIONS

The present study was undertaken to study different factors believed to be of importance for the etiology of obesity. It ought to be pointed out from the beginning that several factors have proven to be of importance for the physiological regulation of body weight and body composition (cf Larsson 1967).

The strain differences in the mice used in the present investigation suggest the importance of hereditary factors for the etiology of obesity. The role of genetic factors for the deposition of fat in adipose tissue has been of importance to those interested in the biology of obesity. In mice, particularly two forms of hereditary obesity have been studied, the so called "yellow obesity" (Danforth 1927) and the hyperglycemic obesity syndrome (cf Myer 1955 and Hellman 1965). In the present study some of the interest has been focused towards the genetical importance upon body composition without definite pathological changes *per se* such as abnormalities in blood glucose in the properties of the pancreas and in the adrenal glands (cf Hellman 1965). Thus in the mice used in the present investigation no signs of inborn abnormalities were observed. Rather one got the impression that external factors such as different types of food or the exercise varied in effect upon body composition due to the hereditary origin.

The relation between body fat and fat free body weight varied in normal individuals due to the strain of mice (Chapter I). This difference could not be due to anything but hereditary differences. It was also found that the Swiss mice after 24 h fast lost more weight relatively compared to the two other strains.

The quality of the protein ingested seems to be of some importance for determining the body fat content. Again the strain characteristics are prominent. The CBA mice seem to be more sensitive to the biological value of the protein than the other strains studied regarding the fat deposition of the body. Thus the lower the protein quality the higher fat content in the body particularly in the CBA mice. This phenomenon is also noted in the other strains but not to the same extent.

When the animals are given different foods comparable to the food eaten by the average Swede and in other groups given the suggested



diet by the National Institute of Public Health it is of interest to note that the revision suggested (Blix *et al* 1965) gives very favorable results regarding low body fat content. In this connection it is worth mentioning that a third diet, low in carbohydrate but high in fat, resulted in extremely high content of body fat and low content of body water which of plain physiological reasons could be considered as not desirable.

The studies on goldthiogluconate obesity reveal that this substance will not behave according to common pharmacological patterns. The production of obesity by this substance is very much due to the amount given but the effect of the substance is due to physiological conditions such as the feeding state of the animal. On the other hand experiments on alloxan diabetic animals tend to show that the reverse situation is active with regard to the toxicity of the substance.

When the mice are exercised the fat content of the body decreases even if the animals are to be regarded as normal because they have not been treated with goldthiogluconate. In obese animals the exercise has a more pronounced effect, because these mice, regardless of strain always loose weight and body fat.

The eating behaviour of the various strains differs significantly. Thus the animals with the highest fat content on the standard diet — the CBA mice — have the lowest frequency of feeding which is in line with similar findings in rats. The choice of diets plays an important role for the body fat content. Thus the mice on free selection of food always have a tendency to be fatter than the corresponding controls.

## SUMMARY

The present experiments have given evidence for the importance and contribution of the following factors for the etiology of obesity in mice

1) *Genetic factors* play an important role since on a given diet strain differences exist with regard to the deposition of fat and the net release of glycerol and FFA from adipose tissue *in vitro*. There is also strain differences in feed utilization and fat deposition when diets with different biological values are studied. Significant strain differences in food preferences were also noted. Pronounced strain differences were found with regard to the sensitivity to goldthioglucose and the incidence of obesity after administration of this substance.

2) *Dietary factors* are also of importance since a food with comparatively low biological value with regard to protein usually leads to low feed utilization and growth. At the same time the body fat content has a tendency to rise. Generally it was noted that the more fat in a diet the higher is the body fat content when the mice are fed *ad libitum*. When the recommendations of the National Institute of Public Health are tried on mice, the body fat content diminishes compared to a diet composed after the average intake of the Swedish people.

3) *Muscular exercise*, even when moderately applied has a profound effect on the body fat content which will decrease. The net release of glycerol and FFA from adipose tissue *in vitro* will increase in exercised animals.

4) *Goldthioglucose obese mice* regardless of the strain all preferred a diet with high fat content when compared to their lean litter mates.

The present study was mainly carried out at the Institute of Physiology, University of Lund.

The work was supported by Statens Medicinska Forskningsrad (projects no 14X 21 02 and B 67 14X 21 03) by Stiftelsen Svensk Näringsforskning and by AB Teknosan Malmö.

To the chief of the Institute Professor George Kahlson and to Professor Dora Jacobsohn thanks are due for all working facilities.

Miss Ulla Hoff has throughout the investigation been extremely skilful and has been unusually devoted to the work.

## REFERENCES

- Adolph E F Urges to eat and drink in rats *Amer J physiol* 1947 151 110—125
- Anand B K, E Dua and B Singh Electrical activity of the hypothalamic feeding centres under the effect of changes in blood chemistry *Electroencephal Clin neurophysiol* 1961 13 54—59
- Anand B K, G S Chhina and B Singh Effect of glucose on the activity of hypothalamic "feeding centres" *Science* 1962 138 597—598
- Altshuler H, M Lieberman and J J Spitzer Effect of body weight on free fatty acid release by adipose tissue *in vitro* *Experientia* 1962 18 91—92
- Andersson B and S Larsson Physiological and pharmacological aspects of the control of hunger and thirst *Pharmacol Rev* 1961 13 1—16
- Anliker J and J Mayer The regulation of food intake. Some experiments relating behavioral, metabolic and morphologic aspects *Amer J clin nutr* 1957 5 148—153
- Barboriak J J, W A Krehl, G R Cowgill and A D Whedon Influence of high fat diets on growth and development of obesity in the albino rat *J Nutr* 1958 64 241—249
- Benjamin W A, A Gellhorn, M Wagner and H Kundel Effect of aging on lipid composition and metabolism in the adipose tissues of the rat *Amer J physiol* 1961 201 540—546
- Blix G A study on the relation between total calories and single nutrients in Swedish food *Acta Soc Med Upsal* 1965 70 117—129
- Blix G, A Wretling, S Bergstrom and S I Westin Kostprogram for en reviderad kost *Näringsforskning* 1965 9 65—72
- Brecher J R and S H Waxler Obesity in albino mice due to single injections of goldthioglucose *Proc soc exp biol med* 1949 70 498—501
- Brobeck J R Neural regulation of food intake *Ann N Y Acad Sci* 1955 63 44—55
- Brobeck J R, Wheatland and J L Strominger Variations in regulation of energy exchange associated with estrus, diestrus and pseudopregnancy in rats *Endocrinology* 1947 4 65—72
- Brobeck J R, S Larsson and E Reyes A study of the electrical activity of the hypothalamic feeding mechanism *J physiol* 1956 133 358—364
- Brožek J Changes of body composition in man during maturity and their nutritional implications *Fed Proc* 1952 11 784—793
- Cham E B, S Larsson and F Pocchiari The fate of glucose in different parts of the rabbit brain *Proc Roy Soc B* 1960 153 283—289
- Chen D B and A B Holt Growth and body composition of the mouse *Amer J physiol* 1963 205 913—918
- Chlouverakis C Adipose tissue lipoprotein lipase activity in rats of two different ages *Nature* 1967 196 1103
- Clausen H *Eur As Animal Prod* Rome 1963
- Clausen H De kvæghaltiga produkternas utnyttjande for proteinsyntesen hos svin *Nord Jordbr forskn* 1963 45 282—297
- Cohn C and D Joseph Changes in body composition attendant on forced feeding *Amer J physiol* 1959 196 965—968
- Cohn C and D Joseph Effects on metabolism produced by the rate of ingestion of the diet *Amer J clin nutr* 1967 8 683—689
- Cohn C and D Joseph Role of rate of ingestion of diet on regulation of intermediary metabolism: meal eating vs nibbling *Metabolism* 1966 9 497—499

- Cohn C D Joseph L Bello and M D Allweiss Studies on the effects of feeding frequency and dietary composition on fat deposition *Ann N Y Acad Sci* 1965 131 507-518
- Danforth C H Hereditary adiposity in mice *J Hered* 1927 18 153-162
- Debons A F L Silver E P Cronkite H A Johnson G Brecher D Tenzer and I L Schwartz Localization of gold in mouse brain in relation to goldthioglucose obesity *Amer J physiol* 1962 202 243-274
- Drachman R H and J Tepperman Aurothioglucose obesity in the mouse *Yale J biol* 1954 26 394-409
- Dury A Relative insensitivity of adipose tissue of old rats to lipolytic action of epinephrine 14th Annual Meeting Gerontology Soc Pittsburgh Pa Nov 1962
- Edelman M I L Schwartz E P Cronkite and L Livingston Studies of the ventromedial hypothalamus with autoradiographic techniques *Ann N Y Acad Sci* 1965 131 485-501
- Ericson, L E S Larsson and G Lid A comparison of the efficiencies of free lysine and of roller dried skim milk fish protein and soya bean protein for the supplementation of wheat bread *Acta physiol scand* 1961 33 366-375
- Ericson L E and S Larsson The loss of added lysine during the baking of a soft bread with an exceptionally high content of reducing sugar *Acta physiol scand* 1962 35 64-73
- Ericson, L E S Larsson and C O Östholm Preliminary studies on the possibility of improving the protein value of cereals for swine by lysine supplementation *Acta agricult scand* 1962a 12 157-166
- Ericson L E S Larsson and S Rubarth The effect of supplementation of wheat bread with amino acids and proteins on liver and muscle composition *Acta agricult scand* 1962b 12 234-250
- Fabry P T Braun R Petrásek E Horáková and E Konopásek The effect of the pattern of food intake on the carcass composition of rats receiving diets with varying fat content *Physiol Bohemoslov* 1964 13 333-339
- FAO Nutrition meeting Report Series No 37 Rome 1965
- Feldman S E, S Larsson M K Dimik and S Lepkovsky Aphagia in chickens *Amer J physiol* 1957 191 259-261
- Fenton P F Studies on genetically determined metabolic processes *Amer J clin nutr* 1960 8 784-751
- Fenton P F and M T Dowling Studies on obesity 1 Nutritional obesity in mice *J Nutr* 1953 49 319-329
- Forsberg A and S Larsson On the central organisation of the nervous mechanism regulating food intake Part II Studies of isotope distribution and chemical composition in the hypothalamic region of hungry and fed rats *Acta physiol scand* 1954 32 suppl 115 41-63
- Forsberg A and S Larsson The feeding centre of the hypothalamic region of the rat brain *Experientia* 1955 11 158
- Friedberg S J N R Harlan D L Trout and E H Estes The effect of exercise on the concentration and turnover of plasma nonesterified fatty acids *J clin invest* 1960 39 215-220
- Fritz, E B D G Davies R H Holthrop and H Dundee Fatty acid oxidation by skeletal muscle during rest and activity *Amer J physiol* 1958 194 379-386
- George J C Muscle lipase *J Anim morphol physiol* 1964 11 234-241
- George J C and N V Vallyathan Capacity for the fatty acid oxidation by the breast muscle of the starling (*Sturnus roseus*) in the pre and postmigratory period *Canad J physiol pharmacol* 1964 42 447-452
- Goodman D S Preparation of human serum albumin free of long chain fatty acid *Science* 1957 125 1296-1297
- Gordon H H Fat Metabolism Ed V A Najar The Johns Hopkins Press 1954
- Hellman B Studies in obese hyperglycemic mice *Ann N Y Acad Sci* 1965 131 541-558
- Hellman B S Larsson and S Westman Acetate metabolism in isolated epididymal adipose tissue from obese hyperglycemic mice at different ages *Acta physiol scand* 1962 36 189-198

## REFERENCES

- Adolph E F** Urges to eat and drink in rats Amer J physiol 1971 181 110-125  
**Anand B h S Dua and B Singh** Electrical activity of the hypothalamic feeding centres under the effect of changes in blood chemistry Electroencephal Clin neurophysiol 1961 13 54-59  
**Anand B k G S Chhinna and B Singh** Effect of glucose on the activity of hypothalamic feeding centres Science 1962 138 537-539  
**Aletschuler H M Lieberman and J J Spitzer** Effect of body weight on free fatty acid release by adipose tissue in the rat Experientia 1962 18 91-92  
**Andersson B and S Larsson** Physiological and pharmacological aspects of the control of hunger and thirst Pharmacol Rev 1961 13 1-16  
**Anliker J and J Mayer** The regulation of food intake Some experiments relating behavioral metabolic and morphologic types Amer J clin nutr 1957 5 148-153  
**Babioriak J J W A Krehl G R Cowall and A D Whedon** Influence of high fat diet on growth and development of obesity in the albino rat J Nutr 1958 64 241-249  
**Benjamin W A A Gelhorn M Warner and H Kundel** Effect of aging on lipid composition and metabolism in the adipose tissues of the rat Amer J physiology 1961 201 540-546  
**Bliz G** A study on the relation between total calories and single nutrients in Swedish food Acta Soc Med Upsal 1960 70 117-129  
**Bliz G A Wretling S Bergström and S I Westin** Nutrition program for cardiovascular disease research Nordisk Medicinskt Forskningsprogram 1960 9 52-72  
**Brecher J P and J H Vahlk** Obesity in albino mice due to single injections of goldfish oil Proc Soc Exp Biol med 1949 70 418-501  
**Brobeck J R** Neural regulation of food intake Ann N Y Acad Sci 1955 63 44-74  
**Brobeck J R Wheatland and J L Strominger** Variations in regulation of energy expenditure associated with estrus diestrus and pseudopregnancy in rats Endocrinology 1964 75 3-12  
**Brobeck J R Lechin and E Reyes** A study of the electrical activity of the hypothalamic feeding center Am J physiology 1966 122 338-364  
**Bucsek J Charney T Haly** Composition in man during maturity and their nutritional requirements J Clin Invest 1951 30 704-713  
**Cannell C and J L Smith** Food intake The effects of glucose in different parts of the rabbit brain Proc Roy Soc Lond Ser B 1961 203 283-289  
**Cannon D T and J H Long** Regulation of food intake by the mouse Am J Physiology 1961 203 7-12  
**Clayton C K Adair** Food intake in rats of two different strains Nature 1962 195 171-172  
**Classen H F Assmann** Fat metabolism in obese subjects J Clin Invest 1963 32 1-12  
**Clausen H L Kyte et al** Neuroendocrine control of lipogenesis in liver and adipose tissue J Clin Invest 1963 32 1-12  
**Coleman C and J L Smith** Food intake The effects of glucose in different parts of the rabbit brain Proc Roy Soc Lond Ser B 1961 203 283-289  
**Coleman C and J L Smith** Food intake The effects of glucose in different parts of the rabbit brain Proc Roy Soc Lond Ser B 1961 203 283-289  
**Coleman C and J L Smith** Food intake The effects of glucose in different parts of the rabbit brain Proc Roy Soc Lond Ser B 1961 203 283-289

- Cohn C D Joseph L Bello and M D Allweiss Studies on the effects of feeding frequency and dietary composition on fat deposition *Ann N Y Acad Sci* 1965 131 507-518
- Danforth C H Hereditary adiposity in mice *J Hered* 1927 18 153-162
- Debons A F L Silver E P Cronkite H A Johnson G Brecher D Tenzer and I L Schwartz, Localization of gold in mouse brain in relation to goldthioglucose obesity *Amer J physiol* 1962 202 243-274
- Drachman R. H and J Tepperman Aurothioglucose obesity in the mouse *Yale J biol* 1954 26 394-409
- Dury A Relative insensitivity of adipose tissue of old rats to lipolytic action of epinephrine 14th Annual Meeting Gerontolog Soc Pittsburgh Pa Nov 1962
- Edelman M I L Schwartz E P Cronkite and L Livingston Studies of the ventromedial hypothalamus with autoradiographic techniques *Ann N Y Acad Sci* 1965 131 485-501
- Ericson L E S Larsson and G Lid A comparison of the efficiencies of free lysine and of roller dried skim milk fish protein and soya bean protein for the supplementation of wheat bread *Acta physiol scand* 1961 53 366-375
- Ericson L E and S Larsson The loss of added lysine during the baking of a soft bread with an exceptionally high content of reducing sugar *Acta physiol scand* 1962 55 64-73
- Ericson, L E S Larsson and C O Ustholm Preliminary studies on the possibility of improving the protein value of cereals for swine by lysine supplementation *Acta agricult scand* 1962a 12 157-166
- Ericson L E S Larsson and S Rubarth The effect of supplementing wheat bread with amino acids and proteins on liver and muscle composition *Acta agricult scand* 1962b 12 234-250
- Fabry P T Braun R Petrasek E Horáková and E Honopásek The effect of the pattern of food intake on the carcass composition of rats receiving diets with varying fat content *Physiol Bohemoslov* 1964 13 333-339
- FAO Nutrition meeting Report Series No 37 Rome 1963
- Feldman S E S Larsson M K Dimik and S Lepkovsky Aphagia in chickens *Amer J physiol* 1957 191 259-261
- Fenton P F Studies on genetically determined metabolic processes *Amer J clin nutr* 1960 8 784-781
- Fenton, P F and M T Dowling Studies on obesity 1 Nutritional obesity in mice *J Nutr* 1953 49 319-329
- Forsberg A and S Larsson On the central organisation of the nervous mechanism regulating food intake Part II Studies of isotope distribution and chemical composition in the hypothalamic region of hungry and fed rats *Acta physiol scand* 1954 32 suppl 115 41-63
- Forsberg A and S Larsson The "feeding centre" of the hypothalamic region of the rat brain *Experientia* 1955 11 158
- Friedberg S J N R Harlan D L Trout and E H Estes The effect of exercise on the concentration and turnover of plasma nonesterified fatty acids *J clin invest* 1960 39 215-220
- Fritz E B D G Davies R H Holtrop and H Dundee Fatty acid oxidation by skeletal muscle during rest and activity *Amer J physiol* 1958 194 379-386
- George J C Muscle lipase *J Anim morphol physiol* 1964 11 234-241
- George J C and N V Vallyathan Capacity for the fatty acid oxidation by the breast muscle of the starling (*Sturnus roseus*) in the pre and postmigratory period *Canad J physiol pharmacol* 1964 42 447-452
- Goodman D S Preparation of human serum albumin free of long chain fatty acids *Science* 1957 115 1296-1297
- Gordon H H Fat Metabolism Ed V A Najar The Johns Hopkins Press 1954
- Hellman B Studies in obese hyperglycemic mice *Ann N Y Acad Sci* 1965 131 541-558
- Hellman B S Larsson and S Westman Acetate metabolism in isolated epididymal adipose tissue from obese hyperglycemic mice at different ages *Acta physiol scand* 1962 56 189-198

- Hellman H S Larsson and S Westman Mast cell content and fatty acid metabolism in the epididymal fat pad of obese mice *Acta physiol scand* 1963 38 255—262
- Hollifield G and W Parson Metabolic adaptations to a stuff and starve feeding program I Studies of adipose tissue and liver glycogen in rats limited to a short daily feeding period *J Clin invest* 1962 41 245—248
- Hollifield G M Perlman and W Parson Free fatty acid content of adipose tissue in three types of obese mice during fasting *Metabolism* 1962 11 117—122
- Ingle D J A simple means of producing obesity in the rat *Proc so exp biol med* 1949 77 604—605
- Jameson G and E Williams The drinking man's diet Cameron Co San Francisco 1964
- Jelinkova M and Z Hruza Decreased effect of norepinephrine and growth hormone on the release of free fatty acids in old rats *Physiol Bohemoslov* 1964 13 327—331
- Kennedy G C The hypothalamus control of food intake II Symposia of the Swedish Nutritional Found Ed G Blin Almquist & Wiksell Solms 1964 56—64
- Keys A Influence of diet on body composition II Symp Swedish Nutr Found Ed G Blin Almquist and Wiksell Stockholm 1964 85—92
- Krook L S Larsen and J Rooney The interrelationship of diabetes mellitus obesity and pyometra in the dog *Amer J vet res* 1960 21 120—124
- Larsson S Food preferences in obesity caused by goldthiogluucose *Acta physiol scand* 1957 40 367—376
- Larsson S A comparison of the biological value of various types of Swedish bread and the effect of added lysine *Acta physiol scand* 1966a 66 157—163
- Larsson S Body protein and fat blood glucose and free fatty acids in various strains of mice *Acta physiol scand* 1966b 68 43—47
- Larsson S Biochemistry of feeding responses and mechanisms *Handbook of Physiology* Amer Physiol Soc Alimentary Canal 1967
- Larsson S and L Strom Some characteristics of goldthiogluucose obesity in the mouse *Acta physiol scand* 1957 38 298—308
- Larsson S T Nilsson and B Olsson Some aspects on the metabolic effect of amino acid supplementation of pig diets *Acta vet scand* 1966 7 47—65
- Laurell S and G Tibbling An enzymatic fluorometric micromethod for the determination of glycerol *Clin Chim Acta* 1966 13 317—322
- Laurell S and C Tibbling Colorimetric micro-determination of free fatty acids in plasma *Clin Chim Acta* 1967 in press
- Leveille G A and R W Hanson Influence of periodicity of eating on adipose tissue metabolism in the rat *Canad J physiol pharmacol* 1965 43 857—868
- Liebelt R A and J H Perry Hypothalamic lesions associated with goldthiogluucose induced obesity *Proc soc exp biol med N Y* 1957 95 774—777
- Liebelt R A, K Sekiba A G Liebelt and J H Perry Genetic susceptibility to goldthiogluucose induced obesity in mice *Proc soc exp biol med N Y* 1960 104 689—692
- Lopez E J L White and F L Engel Contrasting requirements for the lipolytic action of corticotropin and epinephrine on adipose tissue in vitro *J biol chem* 1959 234 2254—2258
- Lowsy O H N J Rosebrough A L Farr and R J Randall Protein measurement with the Folin phenol reagent *J biol chem* 1951 193 265—275
- Lundback K and J A F Stevenson Reduced carbohydrate intake after fat feeding in normal rats and rats with hypothalamic hyperphagia *Amer J physiol* 1947 151 534—537
- Lyon J H and P F Fenton Muscle and liver glycogen of mouse susceptible or resistant to nutritionally induced obesity *Amer J physiol* 1956 187 415—416
- Margolis S and M Vaughan  $\alpha$ -glycerophosphate synthesis and breakdown in homogenates of adipose tissue *J biol chem* 1962 237 44—48
- Marks V An improved glucose-oxidase method for determining blood G S F and urine glucose levels *Clin Chim Acta* 1959 4 395—400
- Marshall N H Influence of epinephrine and fasting on free fatty acid mobilization in goldthiogluucose induced obesity *Proc so exp biol med N Y* 1961 107 941—943

- Marshall N B R J Barnett and J Mayer Hypothalamic lesions in goldthio<sub>2</sub> glucose injected mice Proc. soc. exp biol med 1955 90 243—244
- Martin, D B M G Horning and P R Vagelos Fatty acid synthesis in adipose tissue I Purification and properties of long chain fatty acid synthesizing system J biol chem 1961 236 663—668
- Mažek J and P Fábry High fat diet and the development of obesity in albino rats Experientia 1959 15 444
- Mayer J The regulation of energy intake and the body weight the glucostatic theory and the lipostatic hypothesis Ann N Y Acad Sci 1955 63 15—42
- Mayer J Causative factors in overnutrition II Symp Swedish Nutr Found Fd G Blix Almquist and Wiksell Sto kholm 1964
- Mayer J, N H Marshall J J Vitale J H Christensen M B Mashayekhi and F J Stare Exercise food intake and body weight in normal rats and genetically obese adult mice Amer J physiol 1954 177 544—548
- Michelsen O, S Takahashi and C Craig Experimental obesity J Nutr 1955 57 541—554
- Miller D S and P Mumford, Obesity physical activity and nutrition Proc Nutr soc 1966 25 100—107
- Morrison A B and J A Campbell, Evaluation of protein in foods V Factors influencing the protein efficiency ratio in foods J Nutr 1960 70 112—118
- Moulton C R Age and chemical development in mammals J biol chem 1923 57 79—97
- Munch L The variation of nutritional value in barley II The CBA mouse as test organism for large scale evaluations of nutritional characteristics in programs of plant breeding, Hereditas (Lund) 1964 52 49—58
- Munkner C. *In vitro* production of non esterified fatty acids during the incubation of various types of adipose tissue from fasting and fed rats 1 The mobilization of fatty acids from epididymal perinephric and subcutaneous adipose tissue Scand J clin lab invest 1963 15 83—90
- Oro L L Wallenberg and P Bolme Influence of electrical supramedullary stimulation on the plasma level of free fatty acids blood pressure and heart rate in the dog Acta med scand 1965 178 697—711
- Parizkova J Nutrition and its relation to body composition in exercise Proc Nutr soc 1966 25 93—99
- Parizkova J and L Stankova Influence of physical activity on the metabolism of adipose tissue in rats Brit J Nutr 1964 18 325—352
- van Putten L M D W van Bekkum and A Querido Influence of hypothalamic lesions producing hyperphagia and of feeding regimens on carcass composition in the rat Metabolism 1955 4 68—72
- Raben M S and C H Hollenberg Effect of glucose and insulin on the esterification of fatty acids by isolated adipose tissue J clin invest 1960 39 435—439
- Reinius L Growth failure caused by mild treatment of the diet Acta physiol scand 1955 35 265—271
- Rerup C and I Lundquist, Blood glucose level in mice 1 Evaluation of a new technique of multiple serial sampling Acta Endocrinol (Kbh) 1966 32 357—367
- Richter C P and B Barelare Nutritional requirements of pregnant and lactating rats studied by the selfselection method Endocrinology 1938 2 15—24
- Rushmer R F D L Franklin R L van Citters and O A Smith Changes in peripheral blood flow distribution in healthy dogs Circ Res 1961 9 675—687
- Sebrell W H Metabolic aspects of obesity Metabolism 1957 6 411—416
- Shapiro B I Chowers and G Rose Fatty acid uptake and esterification in adipose tissue Biochim Biophys Acta 1957 23 115—120
- Soyka L F Enhancement of goldthio<sub>2</sub> glucose toxicity and obesity by concurrent glucose administration Toxicol Appl Pharmacol 1966 8 44—50
- Steinberg D and M Vaughan Metabolic and hormonal regulation of the mobilization of fatty acids from adipose tissue In Biosynthesis of Lipids Proc 5th Intern Congress Biochemistry Ed G Popjak London Pergamon Press 1961 p 162
- Steinberg D and M Vaughan Release of free fatty acids from adipose tissue *in vitro* in relation to rate of triglyceride synthesis and degradation Handbook of Physiology Sect 5 Adipose tissue Amer Physiol Soc. 1965 335—347



- Strominger J L, J R Brobeck and R L Cort, Regulation of food intake in normal rats and in rats with hypothalamic hyperphagia Yale J biol med 1953 26 55—74
- Swartz H A, J E Christian and F N Andrews Distribution of sulfur 35 and gold 198 labeled goldthioglucose in mice Amer J physiol 1960 199 67—70
- Taller H, Calories dont count Simon and Shuster N Y 1961
- Tepperman H M and J Tepperman Adaptive hyperlipogenesis Fed Proc 1964 23 73—75
- Tepperman J and H M Tepperman Effect of antecedent food intake pattern on hepatic lipogenesis Amer J physiol 1958 193 55—64
- Tepperman J and H M Tepperman Adaptive hyperlipogenesis — Late 1964 Model Ann N Y Acad Sci 1965 131 404—411
- Tepperman J, J R Brobeck and C N H Long The effects of hypothalamic hyperphagia and of alterations in feeding habits on the metabolism of the albino rat Yale J Biol Med 1943 15 855—874
- Tollersrud S Forsok med lysin tillskudd till slaktegrisar Nord vet med 1961 13 242—258
- Vaughan M The production and release of glycerol by adipose tissue incubated in vitro J biol chem 1962 237 3354—3358
- Wieland O and M Suyter Glycerokinase isolation and properties of the enzyme Biochem Zschrft 1957 329 320—331





ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 205

S. M. S. Lib. 1967

Lib. 1967

Acc. No.

RESERPINE-RESISTANT UPTAKE  
OF CATECHOLAMINES IN  
ISOLATED TISSUES OF THE RAT

*A histochemical study*

By

BERTIL HAMBERGER

STOCKHOLM 1967



ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 295

FROM THE DEPARTMENT OF HISTOLOGY KAROLINSKA INSTITUTET  
STOCKHOLM SWEDEN

RESERPINE RESISTANT UPTAKE  
OF CATECHOLAMINES IN  
ISOLATED TISSUES OF THE RAT

*A histochemical study*

*By*

BERTIL HAMBERGER

STOCKHOLM 1967

ISAR HÄGGSTRÖMS TRYCKERI AB  
STOCKHOLM 196

*To the memory of  
Nils Åke Hillarp*





# CONTENTS

CHAPTER I	
<i>Introduction</i>	7
CHAPTER II	
<i>Methodology</i>	9
A <i>In vitro</i> technique	9
B Histochemical fluorescence method	11
C Standardization of paraformaldehyde	14
D Drugs	19
CHAPTER III	
<i>Uptake of catecholamines in vitro</i>	21
A Localization of endogenous catecholamines in the relevant tissues of untreated animals	21
B Depletion of endogenous catecholamines	23
C Uptake of various catecholamines after reserpine pretreatment	25
D Characterization of reserpine resistant uptake of catecholamines	31
E Effects of drugs on the uptake of catecholamines	34
F Accumulation of catecholamines in the endothelial wall	37
CHAPTER IV	
<i>General Discussion</i>	41
<i>Summary</i>	47
<i>Acknowledgements</i>	48
<i>References</i>	49
<i>Figures</i>	

## KEY TO THE TABLES

Number of experiments within brackets

In our tables the specific fluorescence intensity in the nerve fibres is denoted by the symbols

+++ = strong ++ = moderate + = weak 0 = none

+++ represents the highest fluorescence intensity obtained in the tissues after incubation with C<sub>1</sub> which corresponds roughly to the fluorescence intensity in an untreated animal. Comparisons should be made only between experiment and corresponding control or between experiments in the same series. The difference between *e.g.* ++ and + is distinctly observable. Due to a certain variation in preparations treated identically it was sometimes necessary to bracket the last +. The number of + signs is not quantitatively related nor directly related to a given amount of monoamine or L dopa.

The fluorescence intensity in the endothelial wall is denoted as + (+) or 0.

## INTRODUCTION

It is now generally accepted that NA<sup>1</sup> is the transmitter released from sympathetic adrenergic neurons of mammals (see Euler 1956 Iversen 1967). In view of the presence in the brain of NA and DA in neurons with the same characteristics as sympathetic adrenergic neurons it is probable that the CA serve as transmitters in the central nervous system as well (Carlson Falck and Hillarp 1962 Hillarp Fuxe and Dahlström 1966a b).

The CA neurons in the peripheral and central nervous systems have synthesizing storing releasing and re-transporting properties in addition to several inactivation mechanisms for the transmitter NA released from adrenergic nerves as well as circulating CA can be inactivated in several ways i.e. by diffusion of transmitter from the site of release by extraneuronally located enzymes such as catechol O methyl transferase and MAO by binding to extraneuronal sites and finally—and perhaps most important—by re-uptake into the monoamine neurons (Axelrod 1964 Carlson 1965 Iversen 1965b 1967).

The uptake of CA in tissues has been subjected to extensive studies using isotope techniques and biochemical assays. In adrenergic nerves two levels of uptake are known to exist. One is localized to the level of the cell membrane and is insensitive to reserpine (Hamberger Malmfors Norberg and Sachs 1964 Lindmar and Muscholl 1964 Carlsson and Waldeck 1965 Malmfors 1965 Norberg 1965, Carlsson 1966). A second intraneuronal uptake occurs in amine granules by means of a  $Mg^{++}$  ATP dependent and reserpine sensitive uptake storage mechanism (see Carlsson, Hillarp and Waldeck 1963 Stjärne 1964 Carlsson 1965 Dahlström Fuxe and Hillarp 1965 Euler 1966).

*In vivo* studies on the uptake of CA in the monoamine neurons of the central nervous system are difficult because the amines do

*Abbreviations used* CA = catecholamine(s) NA = noradrenaline DA = dopamine dopa = 3,4-dihydroxyphenylamine MAO = monoamine oxidase

## KEY TO THE TABLES

Number of experiments within brackets

In some tables the specific fluorescence intensity in the nerve fibres is denoted by the symbols

+++ = strong ++ = moderate + = weak 0 = none

+++ represents the highest fluorescence intensity obtained in the tissue after incubation with CA which corresponds roughly to the fluorescence intensity in an untreated animal. Comparisons should be made only between experiment and corresponding control or between experiments in the same series. The difference between e.g. ++ and + is distinctly observable. Due to a certain variation in preparations treated identically it was sometimes necessary to bracket the last +. The number of + signs is not quantitatively related nor directly related to a given amount of monoamine or L-dopa.

The fluorescence intensity in the endothelial wall is denoted as + (-) or 0.

## Chapter II

# METHODOLOGY

### A *In vitro* technique

#### *Procedure*

**ANIMALS** Male albino rats (Sprague Dawley), weighing 150—220 g were used. The rats were sacrificed under light ether anesthesia by exsanguination. The brain and vas deferens were immediately dissected out and placed in ice chilled medium.

**MEDIA** Generally Krebs-Ringer bicarbonate buffer (Umbreit, Burris and Stauffer 1951) was used in which the calcium concentration was lowered by 50% (Axelrod, Gordon, Hertting, Kopin and Potter 1962).<sup>1</sup> The buffer was saturated with 93.5% O<sub>2</sub> and 6.5% CO<sub>2</sub> prior to the experiments and glucose was added to a final concentration of 10 mM. Other media used were Krebs-Ringer phosphate buffer (Umbreit, Burris and Stauffer 1951) and 0.3 M sucrose. The ionic composition of the Krebs-Ringer bicarbonate buffer was changed in some experiments and all ions were replaced by 0.3 M sucrose in others (see Chap. III, D). Glucose was sometimes excluded from the medium and in some experiments nitrogen atmosphere was used in the Krebs-Ringer bicarbonate (N<sub>2</sub> + CO<sub>2</sub>) or phosphate (N<sub>2</sub>) buffer (see Chap. III, D). To media containing CA or dopa small amounts of ascorbic acid (0.2 mg/ml) and EDTA (0.05 mg/ml) were added.

**PREPARATION AND INCUBATION OF SLICES** After chilling the tissue in the medium for at least a few minutes it was put on a moistened filter paper placed on a hard plastic plate on top of a metal block surrounded by ice. Thin slices were prepared with a special carefully cleaned razor blade (Valeport blade strips Gillette) and a frosted object slide mainly according to McIlwain.

<sup>1</sup> Composition of the Krebs-Ringer bicarbonate buffer in g per liter: NaCl 6.923, KCl 0.351, CaCl<sub>2</sub> 6H<sub>2</sub>O 0.28, K<sub>2</sub>HPO<sub>4</sub> 0.16, MgSO<sub>4</sub> 7H<sub>2</sub>O 0.294, NaHCO<sub>3</sub> 0.100.

and Rodnight (1962) The slices of *vis deflexus* were taken along the organ and were 3–5 mm long Coronal slices of the cerebral cortex sagittal slices of the nucleus caudatus putamen and transverse slices of the hypothalamic area were prepared The slices were placed in the medium for not more than 15 min at 0°C and then moved to 50 ml incubation flasks containing 15 ml of medium Generally, two slices from the same tissue were placed in each incubation flask The flasks were gassed and rubber stoppered and then incubated at 37°C under continuous shaking In the experiments with nitrogen atmosphere the flasks were gassed continuously When the uptake after CA or dopa was studied the slices were as a rule first preincubated for 15 min before addition of CA or dopa When the influence of drugs on the accumulation of CA was studied these drugs were always present during preincubation When the effect of exclusion of glucose was studied glucose was also excluded from the preincubation medium Unless otherwise stated, the incubation time was 20 to 30 min after the addition of CA or dopa With longer incubation times the flasks were regassed every hour Controls generally from the same rat as that in the experiments were included The controls were incubated in Krebs Ringer bicarbonate buffer without drugs for the same time as in the experiment and in uptake inhibition experiments uptake controls were also included

### Comments

The *in vitro* technique with incubation of tissue slices has earlier been used under a number of experimental conditions (see e.g. Field 1948 Mellin and Rodnight 1962) In order to preserve the tissues and make especially the brain easier to slice the tissues were immediately immersed in ice-chilled medium The crucial factor is to prepare uniform slices which are thin enough to allow diffusion of substrates and oxygen throughout them In practice the most critical point is to maintain adequate oxygenation The thickness of e.g. a rat cerebral cortex slice should not be more than 0.6 mm (Field 1948) The thickness of the rat cortex outside the white matter is about one mm on the top of the skull (König and Klippel 1963) When two coronary slices were made the second regularly just reached the white matter which implied

that the thickness of the cortical slice was about 0.5 mm or slightly more. In other central areas the slices were of about the same thickness. Vas deferens slices were easy to cut thinner i.e. about 0.3 mm (approximated from the total thickness of the vas deferens and the number of slices obtained).

The use of the histochemical method provides a possibility of judging the vitality of the tissue, and checking the preparative procedure. The accumulation of CA in a nerve terminal suggests that both CA and the substrate for energy generation have reached the terminal since the uptake is energy dependent (cf Chap III, D). In slices of the vas deferens cerebral cortex and hypothalamus the distribution of fluorescent nerve fibres after reserpine resistant accumulation of CA was almost invariably homogeneous throughout the section. In the nucleus caudatus putamen however a completely different picture was found. A fluorescent zone was often seen in the border of the slice whereas the interior showed no specific fluorescence even after high concentrations of CA. When evaluating slices from the nucleus caudatus putamen only the outer border was therefore taken into account.

The quantitative preservation of NA in the incubation bath at 37°C under the present conditions i.e. with small amounts of ascorbic acid and EDTA was studied (Haggendal and Hamburger 1967). It was shown that under the relevant conditions about 90% of the added NA was unchanged after one hour.

## **II Histochemical fluorescence method**

### *Procedure*

After incubation the slices were placed on small pieces of filter paper and attached by a drop of serum from the animal from which the tissue was taken and immediately frozen in propane chilled with liquid nitrogen. The tissue was then freeze dried in a high capacity freeze drier<sup>1</sup> designed by Thieme (1965). The frozen tissue was rapidly moved directly from the propane to the cold plate of the freeze drier and placed in pulverized CO<sub>2</sub> ice with which the cold plate was covered. During the first hour CO<sub>2</sub> ice was also placed on top of the freeze drying chambers as a cold trap. The freeze drier was generally run for at least 3 h

<sup>1</sup>The freeze-drier is manufactured by HETO, Birkedal, Denmark.



with the lowest obtainable temperature in the cold plate ( $-40^{\circ}$  to  $-50^{\circ}\text{C}$ ). The temperature was then raised to about  $-30^{\circ}\text{C}$  for 6–12 h, whereafter the cold plates were allowed to warm up for at least 2 h before removing the specimens. These were treated with formaldehyde gas generated from paraformaldehyde at  $80^{\circ}\text{C}$  for 1 h. All tissue specimens from one experiment were always treated with formaldehyde gas in the same reaction vessel. For preparation of paraformaldehyde see Chap. II C. The slices which at this stage were extremely delicate were embedded in paraffin, sectioned at  $8\text{ }\mu$  and mounted in Entellan (Merck) containing a small amount of xylene.

For microscopy, a fluorescence microscope (Zeiss Standard Junior) with non fluorescent objectives and a dark field oil condenser was used. The light source was a HBO 200 high pressure mercury lamp (Osram) with a 3 or 4 mm Schott BC 12 filter a Zeiss 50 (2 mm) being used as secondary filter. Scopix G (Gevaert) was used for microphotography. For details of the technique and optical equipment see also Dahlström and Fuxe (1964), Norberg and Hamberger (1964), Falck and Owman (1965) and Malmfors (1965).

### *Comments*

The chemistry and specificity of the present histochemical technique have been discussed and established in several papers (Falck 1962, Falck, Hillarp, Thume and Torp 1962, Corrodi and Hillarp 1963, 1964, Corrodi, Hillarp and Jonsson 1964, Corrodi and Jonsson 1965, 1966, 1967, Caspersson, Hillarp and Rützen 1966, Jonsson 1966, 1967 a, b, c, Rützen 1966, 1967). There is thus little doubt that the specific yellow green fluorescence (with the fluorescence microscopical equipment used) in nerve cell bodies, non terminal axons and terminals of peripheral tissues (Norberg and Hamberger 1964, Malmfors 1965, Norberg 1967) and of central tissues (Dahlström and Fuxe 1965, Fuxe 1967 a, b, Hillarp, Fuxe and Dahlström 1966 a, b) from untreated animals is derived from the 6,7-dihydroxy-3,4-dihydroquinolines which are formed from the endogenous CA and formaldehyde gas. In the experiments where the endogenous monoamines were depleted with a large dose of reserpine the specific yellow green fluorescence found after incubation with NA, DA or dopa was derived analogously.

from the corresponding 3,4 dihydroisoquinolines. Dopamine may have been converted to DA or NA and this will be discussed separately. Also DA (or  $\alpha$  methyl DA) may have been converted to NA (or  $\alpha$  methyl NA).  $\alpha$  Methyl NA has been shown to form 6,7 dihydroxy 3,4 dihydroisoquinolines with practically the same fluorescence yield as NA and DA while  $\alpha$  methyl DA gives a lower yield (Jonsson 1967a). The 3-O-methylated metabolites of NA or DA do not form strongly fluorescent products with formaldehyde gas (Jonsson 1966, 1967a) and can be concluded not to interfere markedly under the present conditions. Furthermore, the demethylated metabolites do not react with formaldehyde.

When evaluating the results of changes in the fluorescence intensity (Chap. III) the material to be compared was always freeze dried on the same occasion and treated with formaldehyde gas in the same reaction vessel. The intensity and distribution of the specific fluorescence in the experimental material were estimated in the microscope and compared to those of controls. The fluorescence intensity and the morphological quality could slightly vary between the separate experimental series in all probability on technical grounds.

Model experiments have shown that CA concentration is proportional to fluorescence intensity below a certain concentration and that a further rise in CA concentration gives an unchanged or even slightly diminished intensity (Ritzen 1966) due to quenching of the fluorescence. The concentration for the occurrence of quenching in models is about 8000  $\mu\text{g/g}$  wet weight (Ritzen 1966). The average NA concentration in e.g. the peripheral adrenergic terminal has been estimated to be 1000–3000  $\mu\text{g/g}$  wet weight (Dahlström, Haggendal and Hokfelt 1966) but the local concentration of NA may well be even higher. As regards DA terminals of the nucleus caudatus putamen the average concentration of DA in the varicosities was suggested to be even higher 8000  $\mu\text{g/g}$  wet weight (Anden, Luxe, Hamberger and Hokfelt 1966). Regardless of previous treatment of the tissue it is however difficult to draw any reliable conclusions about the subcellular distribution of the fluorescent products after freeze drying and formaldehyde gas treatment (Ritzen 1967) which makes it difficult to evaluate when quenching occurs in tissue experiments.

The general experience is that observed increases and decreases in fluorescence intensity reflect corresponding changes in mono-

amine levels (cf Corrodi and Jonsson 1967) Such changes in fluorescence intensity are evidently observable after partial depletion of the normal CA stores or accumulation of CA after *e.g.* reserpine induced depletion of the endogenous monoamines (Malmfors 1965 Norberg 1965 Hillarp Lave and Dahlstrom 1966 b Corrodi and Jonsson 1967) Under the present conditions the reproducibility of the method was found to be satisfactory which implies that the fluorescence intensity can safely be regarded as a semiquantitative measure of changes in CA content

### C. Standardization of paraformaldehyde<sup>1</sup>

#### *Procedure*

The water content of paraformaldehyde (Walker 1964) was determined by the Karl Fischer method (Fischer 1935 Mitchell and Smith 1948 Muroi and Ogawa 1963 Information from Pharmacia Uppsala Sweden) A Karl Fischer electrometric titration apparatus operating by the dead stop method was used (Type F 297, Metrohm AG Herisau Switzerland) 100 to 250 mg of paraformaldehyde (purum Merck) were extracted in at least 15 ml of water free methanol for 10 min and then titrated with a stable Karl Fischer reagent in one solution (Pharmacia Uppsala Sweden) The titre of the reagent was about 4 mg  $H_2O$  per ml and was regularly controlled by titration of measured amounts of sodium tartrate (p.a. Riedel de Haen  $Na_2C_4H_4O_6 \cdot 2H_2O$ ) At least two determinations were made on each paraformaldehyde

To obtain paraformaldehyde with varying water content it was equilibrated at room temperature in closed vessels containing aqueous solutions of sulphuric acid of varying density (Table I)

#### *Experiments on paraformaldehyde*

The sensitivity and reproducibility of the present titrimetric method were tested by measuring the water content of 10 samples from the same paraformaldehyde (Table II)

The paraformaldehyde used for the equilibration experiments

<sup>1</sup> A preliminary report has been given of some of the present experiments (Hamberger Malmfors and Sachs 1967)

TABLE I Relation between relative humidity density and g concentrated sulphuric acid per litre for aqueous solutions of sulphuric acid

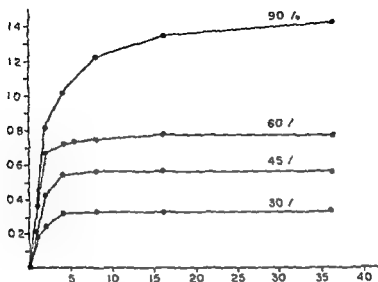
Relative humidity %	Density	Concentrated sulphuric acid g/l
10	1.59	1061
20	1.49	897
30	1.41	779
40	1.39	678
50	1.34	586
60	1.29	503
70	1.25	428
80	1.20	341
90	1.14	241

<sup>1</sup> From Handbook of Chemistry and Physics 30th Edition Chemical Rubber Publishing Co. Cleveland 1947 pp 1635-19.6

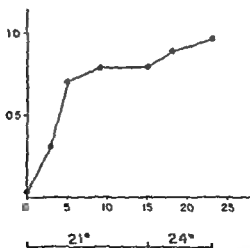
TABLE II Water adsorbed in 10 samples of the same paraformaldehyde equilibrated in 50 per cent relative humidity

Sample	% water
1	0.500
2	0.503
3	0.504
4	0.553
5	0.561
6	0.557
7	0.556
8	0.550
9	0.561
10	0.560

had been dried over phosphorous pentoxide for at least one week. Such paraformaldehyde has a water content below 0.05 per cent. The dried paraformaldehyde was equilibrated at room temperature in closed vessels with varying relative humidity obtained from aqueous solutions of varying density (Table I). The result of a typical experiment shows that paraformaldehyde slowly adsorbed water from the atmosphere until it reached a certain level which was dependent on the relative humidity in the closed vessel (Text fig. I). As a rule the water content of the paraformal



Test fig. I Water content of paraformaldehyde equilibrated at room temperature in closed vessels with different constant relative humidity. Dried paraformaldehyde was used. The relative humidity was obtained from an aqueous solution of sulphuric acid (see table I).



Test fig. II Water content of paraformaldehyde equilibrated in a closed vessel with 70% relative humidity obtained from an aqueous solution of sulphuric acid (see table I). Dried paraformaldehyde was used. The equilibration temperature was 21°C for 15 h, and it was then increased to 24°C.

dehyde then stayed constant for a long time although that equilibrated with 90 per cent relative humidity showed a tendency to increase its water content further in some experiments

The equilibration level was also found to be dependent on the temperature during the experiment. A typical such experiment is seen in Text fig II. After equilibration had been reached at 21°C an increase in temperature to 24°C produced a further increase in water content.

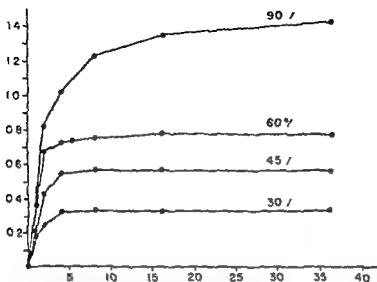
### *Preparation of paraformaldehyde for routine use*

The paraformaldehyde is equilibrated as described above. Although it is not as a rule dried before equilibration a constant water content is obtained within a short time and the paraformaldehyde is generally utilized for histochemical experiments after about 10 days. In the present *in vitro* experiments the water content ranged from 0.5 to 0.8 per cent. The paraformaldehyde was used once only.

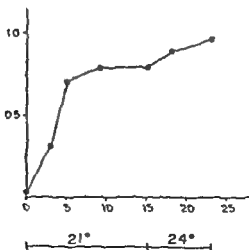
### *Comments*

The sensitivity and reproducibility of the water determination method were found to be satisfactory for the present purpose. Although it has been claimed that formaldehyde itself could interfere when applying the conventional Karl Fischer method this is not generally accepted (Mitchell and Smith 1948). Using the Karl Fischer reagent produced by Pharmacia this interference can be disregarded (Information from Pharmacia Uppsala, Sweden). Furthermore Muroi and Ogawa (1963) found that with their special Karl Fischer reagent no reaction between methanol and formaldehyde occurred below 30°C.

For a satisfactory histochemical result it is necessary for a certain amount of water to be present during the reaction of monoamines with formaldehyde gas while too much water causes diffusion of the monoamines. It has not been fully clarified why a certain amount of water must be present during the reaction although the reaction between CA and formaldehyde is a condensation reaction. The role played by the water is probably due partly to an increased reactivity of the formaldehyde gas but it might also provide a more optimal pH of the surrounding proteins.



Text fig. I Water content of paraformaldehyde equilibrated at room temperature in closed vessels with different constant relative humidity. Dried paraformaldehyde was used. The relative humidity was obtained from an aqueous solution of sulphuric acid see table I



Text fig. II Water content of paraformaldehyde equilibrated in a closed vessel with 70% relative humidity obtained from an aqueous solution of sulphuric acid see table I. Dried paraformaldehyde was used. The equilibration temperature was 21°C. The water content was increased to 24°C

dehyde then stayed constant for a long time although that equilibrated with 90 per cent relative humidity showed a tendency to increase its water content further in some experiments.

The equilibration level was also found to be dependent on the temperature during the experiment. A typical such experiment is seen in Text fig. II. After equilibration had been reached at 21°C an increase in temperature to 24°C produced a further increase in water content.

### *Preparation of paraformaldehyde for routine use*

The paraformaldehyde is equilibrated as described above. Although it is not as a rule dried before equilibration a constant water content is obtained within a short time and the paraformaldehyde is generally utilized for histochemical experiments after about 10 days. In the present *in vitro* experiments the water content ranged from 0.5 to 0.8 per cent. The paraformaldehyde was used once only.

### *Comments*

The sensitivity and reproducibility of the water determination method were found to be satisfactory for the present purpose. Although it has been claimed that formaldehyde itself could interfere when applying the conventional Karl Fischer method this is not generally accepted (Mitchell and Smith 1948). Using the Karl Fischer reagent produced by Pharmacia this interference can be disregarded (Information from Pharmacia Uppsala, Sweden). Furthermore Muroi and Ogawa (1963) found that with their special Karl Fischer reagent no reaction between methanol and formaldehyde occurred below 30°C.

For a satisfactory histochemical result it is necessary for a certain amount of water to be present during the reaction of monoamines with formaldehyde gas, while too much water causes diffusion of the monoamines. It has not been fully clarified why a certain amount of water must be present during the reaction although the reaction between CA and formaldehyde is a condensation reaction. The role played by the water is probably due partly to an increased reactivity of the formaldehyde gas but it might also provide a more optimal pH of the surrounding proteins.



favouring the existence of the quinoidal form of the 6,7-dihydroxy-3,4-dihydroisoquinolines responsible for the strong fluorescence (Corrodi, Jonsson and Malmfors 1966; Ritzen 1967). It is also possible that small amounts of water cause intraneuronal diffusion of the amines (Ritzen 1967).

The water present during the histochemical reaction may derive from the tissue, from the air initially enclosed in the reaction vessel and from the paraformaldehyde. The influence of tissue water after controlled and efficient freeze-drying and proper handling conditions may be considered constant and probably fairly low. Incubation of freeze-dried spinal cord in an atmosphere of high relative humidity has been used to increase the tissue water before the formaldehyde reaction (Dahlstrom and Fuxe 1963) but this method is not in general use.

Water may also derive from the air initially enclosed in the reaction vessel generally with a volume of 1 litre. With 50 per cent relative humidity the air in such a vessel contains about 11 mg of water at 21°C (Handbook of Chemistry and Physics). This water can be controlled and kept on a constant level by using a room with constant relative humidity and temperature.

Generally about 11 g of paraformaldehyde are used. The amount of adsorbed water available in a paraformaldehyde containing 0.8 per cent water would thus be about 50 mg. Judging by the figures of Muroi and Okawa (1963) about the same amount can be derived from the pyrolysis of paraformaldehyde at 50°C for 1 h. Under the histochemical conditions used paraformaldehyde thus seems to be the most important source of water. The water originating from the pyrolysis is probably constant since paraformaldehyde is utilized once only. Under these conditions the variation in adsorbed water is the crucial factor.

For quantitative determinations of catecholamines with the present method it is of great importance to use standardized paraformaldehyde as different humidities give varying fluorescence yields (Norberg, Ritzen and Ungerstedt 1966; Ritzen 1966). Use of paraformaldehyde with a high water content may also induce side reactions under certain conditions (Casperson, Hillarp and Ritzen 1966; Ritzen 1966; Jonsson 1967b).

Paraformaldehyde standardized in the way described has been used in this laboratory for all kinds of tissue since the end of 1963 (cf. e.g. Malmfors 1965; Hillarp, Fuxe and Dahlstrom 1966).

11 b Norberg 1967) The histochemical results appear to be dependent above all on the adsorbed water of paraformaldehyde. Distinct impairment of the histochemical results was also observed when the climate was hot and humid.

The foregoing data emphasize the necessity of controlling and standardizing all steps in the histochemical technique i.e. freeze drying efficiency, handling of tissue before reaction with paraformaldehyde and water content of air and paraformaldehyde. Handling of tissue in a room with low and constant relative humidity and constant temperature is highly desirable. As in addition a certain variation exists between tissues (cf. Dahlstrom and Tuve 1963, Olson 1967) it is advisable to test paraformaldehyde with a water content from 0.3 to 1.2 per cent for practical work.

## D Drugs

The following drugs were injected intraperitoneally:

Reserpine (Serpasil® Ciba) 5–10 mg/kg 12–18 h before killing  
Nialamide (Niamid® Pfizer) 100 mg/kg 1–3 h before killing  
dissolved in 0.9% NaCl with the help of a small amount of N HCl after which the solution was adjusted to about pH 5 with N NaOH.

Unless otherwise stated the following drugs were dissolved in Krebs-Ringer bicarbonate buffer and then diluted in the same buffer. The catecholamines and metaraminol were calculated as the base. The concentrations used are given:

(—) NA (Sigma) 0.01–10 µg/ml

(—)  $\alpha$  Methyl NA hydrochloride (Corbasil® Hoechst) 0.001–10 µg/ml

DA hydrochloride (Sigma) 0.01–10 µg/ml

$\alpha$  Methyl DA hydrochloride (Hassle) 0.01–10 µg/ml

(—) Metaraminol bitartrate (Aramine®, Merck Sharp & Dohme) 0.1–10 µg/ml

L dopa (Sigma) 0.1–100 µg/ml

D dopa (Sigma) 0.1–100 µg/ml

Reserpine phosphate (Ciba)  $10^{-2}$ – $10^{-4}$  M dissolved in 5% glucose

Desipramine (Pertofran® Geigy)  $10^{-2}$ – $10^{-4}$  M

(+) Amphetamine sulphate (Sigma),  $0.8 \times 10^{-4}$  —  $2.7 \times 10^{-4}$  M  
 Chlorpromazine (Hibernal® Leo)  $10^{-6}$  —  $10^{-3}$  M  
 Cocaine hydrochloride  $10^{-6}$  —  $10^{-3}$  M  
 NSD 1015 (3 hydroxybenzyl hydrazine fumarate, Smith and  
 Nephew Research Ltd) 50  $\mu$ g/ml  
 2,4 Dinitrophenol (p.a. Merck)  $5 \times 10^{-4}$  —  $10^{-3}$  M  
 Potassium cyanide (p.a. Merck)  $10^{-3}$  M  
 Ouabain (g Strophantin Sandoz)  $10^{-3}$  M

## UPTAKE OF CATECHOLAMINES IN VITRO

### A Localization of endogenous catecholamines in the relevant tissues of untreated animals

#### *Results*

Slices of four model tissues were used for the present experiments vas deferens cerebral cortex hypothalamus and nucleus caudatus putamen. The fluorescence microscopical appearance of slices from these areas in untreated animals is described in the following.

In slices of the vas deferens a dense network of strongly fluorescent nerve terminals with distinct varicosities was seen within the smooth muscle layers (Figs 1, 3). Bundles of non terminal axons were often present in the outer border of the smooth muscle (Fig 3). The distribution of adrenergic terminals in normal tissues has been thoroughly described earlier (Palck 1962, Norberg and Hamberger 1964, Owman and Sjostrand 1965, Norberg, Risley and Ungerstedt 1967).

In slices of various areas of the cerebral cortex very fine fluorescent terminals (Figs 5, 9) were seen in a network of varying density (cf Fuxe, Hamberger and Hokfelt to be publ.). These terminals are difficult to reproduce regularly unless their fluorescence has been increased by incubation with CA (see below) but have been observed earlier in *in vivo* preparations (Fuxe 1965 b).

Slices of the nucleus caudatus putamen showed a dense network of mainly very fine terminals (cf Figs 6, 8). The single terminals are often difficult to distinguish in *in vivo* preparations (Fuxe, Hokfelt and Nilsson 1965) but can be clearly seen after incubation without CA in the medium. The single terminals are especially easy to distinguish in 1–2  $\mu$  thick Araldite sections after incubation with  $\alpha$  methyl NA (Anden, Fuxe, Hamberger and Hokfelt 1966).

The microscopical appearance of slices from the hypothalamic area was generally less uniform than that just described. A network of fine to fairly thick terminals of varying density was

always present (Fig. 10). The distribution of CA nerve terminals has been thoroughly mapped out (Tuxe 1965 a, b).

Incubation of slices from the aforementioned areas in the standard Krebs Ringer medium did not decrease the fluorescence intensity even if incubation was continued for 2 h. In a few experiments slices of vas deferens were incubated for 2 h and no decrease in fluorescence was apparent.

Incubation of slices from untreated rats with NA up to 1  $\mu\text{g/ml}$  did not markedly change the appearance, whereas a larger dose (10  $\mu\text{g/ml}$ ) resulted in increased fluorescence (cf. Angelakos and King 1965). Pretreatment with malamide (100 mg/kg) before incubation with NA or  $\alpha$ -methyl NA increased the fluorescence at 1  $\mu\text{g/ml}$  as well. After this treatment the bundles of non-terminal axons in slices of the vas deferens showed increased fluorescence (cf. Fig. 4). Non-terminal axons both in large bundles and singly were also observed in slices from the hypothalamic area (Figs. 12, 13). In slices of the cerebral cortex (Fig. 9) and nucleus caudatus putamen the fluorescent nerve terminals were more distinct after incubation with e.g.  $\alpha$ -methyl NA.

Furthermore incubation of brain slices with CA or L-dopa in a concentration of 1  $\mu\text{g/ml}$  or more resulted—provided that the effect of MAO had been excluded—in strong fluorescence in the walls of the capillaries, pericytes and/or endothelial cells (Hamberger and Masuoka 1965). The fluorescence in these cells occurred in brain slice of both untreated and reserpine pretreated rats (Figs. 18 to 21) and will be described separately in Chap. III F.

## Discussion

The reason for which these experimental tissues were chosen was to obtain representative tissues which are relatively homogeneous with respect to CA content and large enough to provide several slices from each animal. It is also highly desirable that the NA and DA nerve terminals can be studied separately. The smooth muscle layer of the vas deferens (Falck 1962; Norberg and Hamberger 1964; Owman and Sjöstrand 1965; Norberg, Risley and Ungerstedt 1967) and the cerebral cortex (see Anden, Tuxe and Ungerstedt 1967) contain NA terminals. The nucleus caudatus putamen has large numbers of DA nerve terminal (see Anden, Tuxe, Hamber-

ger and Hokfelt 1966) In the hypothalamus the predominant CA is NA which implies that most terminal and non terminal axons normally contain NA (see Fuxe 1965 b) However, DA nerve terminals and non terminal axons are also present in this area e.g. the tubero infundibular DA neurons (Fuxe 1964 see also Chap III F)

Incubation of slices from untreated animals with  $\alpha$  methyl NA was used for thorough studies of the appearance and distribution of CA terminals in several areas This is of special value when it is normally difficult to detect the individual terminal, as in the cerebral cortex (Fuxe Hamberger and Hokfelt to be publ), the nucleus caudatus putamen (Anden Fuxe, Hamberger and Hokfelt 1966) and the brown adipose tissue (Wirsén 1965 Wirsén and Hamberger 1967)

## B Depletion of endogenous catecholamines

### Results

The ability of reserpine and desipramine in varying concentrations to deplete the endogenous CA *in vitro* was investigated The effect of reserpine phosphate on vas deferens slices of untreated rats is seen in Table III A concentration of  $10^{-5}$ M produced a distinct decrease in the fluorescence intensity after 1 h while  $10^{-6}$ — $10^{-9}$ M had a roughly similar effect after 2 h Specific fluorescence was still observed after incubation for 4 h with reserpine If the tissue had been exposed to reserpine only for the first 15 min of the total incubation time the decrease was the same as if reserpine had been present throughout incubation  $10^{-5}$ M reserpine phosphate induced a prominent yellow background fluorescence which could be faded by the activation light (Haycock Sheth and Mader 1959)

The effect of desipramine on the vas deferens hypothalamus and nucleus caudatus putamen *in vitro* is shown in Table IV Desipramine in a high concentration ( $10^{-5}$ M) had the ability to decrease fluorescence intensity from endogenous CA highly efficiently no specific fluorescence was found after 15 min  $10^{-6}$ M desipramine and lower concentration did not appear to have any such effect

TABLE III Fluorescence intensity in vas deferens nerve terminals of untreated rats after incubation with reserpine

Reserpine concentration	Incubation time			
	30 min	60 min	120 min	240 min
10 <sup>-6</sup> M	++(+)(3) <sup>1</sup>	+ (5) <sup>1</sup>	(+) (5) <sup>2</sup>	(+) (3) <sup>2</sup>
10 <sup>-5</sup> M	+++ (3)	+(+)(4)	+ (4)	(+) (3)
10 <sup>-4</sup> M	+++ (3)	++ (5)	+ (5)	(+) (3)
10 <sup>-3</sup> M	+++ (3)	++(+) (4)	+ (4) <sup>2</sup>	(+) (3)
10 <sup>-2</sup> M	+++ ( )	++(+) (5)	+(+) (5)	+ (3)
0	+++ (8)	+++ (9)	+++ (6)	+++ (3)

For symbols used see key to the tables p 6

Reserpine phosphate was dissolved in a 5% glucose and diluted in Krebs-Ringer bicarbonate medium

<sup>1</sup> Prominent yellow background fluorescence

<sup>2</sup> Same result when exposure time to reserpine was 15 min followed by incubation of the tissue in medium without drugs (3)

TABLE IV Fluorescence intensity in nerve terminals of vas deferens hypothalamus and nucleus caudatus putamen<sup>1</sup> of untreated rats after incubation with desipramine

Desipramine concentration	Incubation time			
	10 min	30 min	60 min	120-180 min
10 <sup>-6</sup> M	0 (3)	0 (3)		
10 <sup>-5</sup> M		+ (3)	0 (3)	
10 <sup>-6</sup> M — 10 <sup>-5</sup> M		+++ (3)	+++ (3)	+++ (3)
0		+++ (6)	+++ (6)	+++ (5)

For symbols used see key to the tables p 6

<sup>1</sup> Two experiments on nucleus caudatus putamen at each point

## Discussion

Reserpine is known to prevent the accumulation of CA in amine granules by interfering with the  $Mg^{++}$  ATP dependent uptake storage mechanism in the granules and cause the endogenous CA to disappear (see Carlsson Hillarp and Waldeck 1967 Fulcr and Ishayko 1963 Dohl from Fuxe and Hillarp 1965 Carlsson 1965). The reserpine induced depletion of endogenous NA was slow even slightly slower than in *in vivo* experiments since detectable amounts were found also after 6 h. This could be partly due to

reduction in nervous activity which has been shown to slightly delay the depletory effect of reserpine (Sedvall 1964 Malmfors 1965, Anden Tuxe and Hokfelt 1967)

Exposure of the tissue to reserpine for 15 min only caused the same depletion as if reserpine was present during the whole incubation time. This suggests that the interference of reserpine with the uptake storage mechanism has a rapid onset and that the effect cannot be reversed by removing reserpine from the incubation medium (cf Dahlstrom and Haggendal 1966)

The depletory effect of desipramine is seen with  $10^{-4}$ M or higher concentrations while with  $10^{-5}$ M no depletion is observed. It has also been reported that perfusion of cat hearts with high concentrations of desipramine (above  $10^{-4}$ M) results in a rapid large release of endogenous NA (Titus Matussek, Spiegel and Brodie 1966). *In vivo* desipramine in moderate doses does not lower endogenous CA (Carlsson 1966). Recent data have shown that high doses of desipramine and protriptyline can, in fact, lower the endogenous CA levels in the brain (Carlsson pers. comm.). A probable reason for the depletory action of desipramine is blockade of the  $Mg^{++}$  ATP dependent uptake storage mechanism in amine granules which at least in adrenal medullary granules occurs in concentrations above  $10^{-5}$  M (Carlsson, Hillarp and Waldeck 1963). The very fast effect at  $10^{-3}$ M suggests that at this concentration desipramine directly interferes with the storage of CA.

As reported in Chap III E, desipramine prevents accumulation of CA in NA but not in DA terminals but its depletory effect seems equally good in both. It thus seems probable that the depletory effect of desipramine on endogenous CA differs from the inhibition of uptake in NA terminals.

### C. Uptake of various catecholamines after reserpine pretreatment

#### *Results*

All rats used in the experiments were pretreated with reserpine (5 to 10 mg/kg) and killed 12 to 18 h later, and some were



pretreated with mianserin (100 mg/kg) 1—2 h before killing. Control tissues were taken from all animals. After this treatment no specific fluorescence of CA could be detected. The restitution of specific fluorescence in nerve terminals and non terminal axons after incubation of slices with CA or DA was investigated.

The restitution of specific fluorescence in the vas deferens and cerebral cortex was studied after different incubation times. The slices were preincubated for 15 min in the Krebs Ringer bicarbonate buffer after which  $\alpha$ -methyl-NA was added to a final concentration of 1.01 or 0.01  $\mu$ g/ml. The incubation time was 5, 20, 60 or 120 min. The restitution in nerve terminals after 20 min is seen in Table V. The longer incubation times did not increase the fluorescence intensity appreciably while incubation for 5 min resulted in considerably lower intensity than incubation for 20 min.

In the four model tissues both NA and DA restored the specific fluorescence in the nerve terminals provided that the rats had been pretreated with the MAO inhibitor mianserin or their  $\alpha$ -methylated analogues (Figs. 2, 4 to 6, 9, 15). The nerve terminals showed fluorescent varicosities. It can be inferred from Table V that the accumulation as reflected in fluorescence intensity of the nerve terminal was in general concentration dependent in the concentration range used. This also applied to non terminal axons (Fig. 2, 4, 7, 14, 15) which after this treatment exhibited fluorescence similar to that of the nerve terminals. Strongly fluorescent cell bodies were also occasionally observed (Fig. 11). Incubation with 10  $\mu$ g/ml CA produced general background fluorescence. Incubation of slices from the vas deferens or cerebral cortex with NA without pretreatment with a MAO inhibitor resulted in only weak fluorescence in nerve terminal and non terminal axons even at high NA concentrations. This fluorescence could be abolished if the tissue was rinsed in the Krebs Ringer bicarbonate buffer without NA for 15 min whereas the fluorescence induced by  $\alpha$ -methyl-NA or by NA after pretreatment with MAO inhibitor did not diminish after such rinsing.

In many fibres from the hypothalamic area bundles of strongly fluorescent non terminal axons were visible after incubation with CA (Fig. 14). In view of their localization (see Chap. III A) and the pharmacological results (see Chap. III I) part of these non terminal axons in all probability belong to DA neurons. As

TABLE V Fluorescence intensity in nerve terminals after incubation of slices from re-erpine pretreated rats with CA

Drugs	Concentration μg/ml	Tissue		Cerebral cortex	Hypo- thalamus	Nucleus caudatus putamen
		Vas deferens				
NA	10	++	(3)	+(+)	(2)	DA 10 +++(+) (2)
	1	+	(4)	(+)	(7) <sup>1</sup>	DA 1 +++ (2)
	0.1	0	(3)	0	(3)	DA 0.1 (+) (2) <sup>1</sup>
Nialamide + NA	10	+++	(2)	++(+)	(4)	+++ (+)
	1	++(+)	(4)	+	(4)	+++ (4)
	0.1	+(+)	(3)	+	(2)	+(+) (3)
	0.01	(+)	(3)			
α Methyl NA	10	+++	(6)	++(+)	(4)	
	1	++(+)	(3)	++	(16)	+++ (14)
	0.1	++	(16)	+	(12)	+(+) (13)
	0.03	+	(9)			
	0.01	+	(8)	0	(2)	+
	0.003	(+)	(7)			
	0.001	0	(7)			
Nialamide + DA or α methyl DA	10	++(+)	(2)	++(+)	(4)	+++ (4) <sup>2</sup>
	1	++(+)	(8)	++	(5)	+++ (7) <sup>2</sup>
	0.1	+(+)	(2)	+	(4)	+(+) (4)
	0.01			0	(2)	+

For symbols used see key to the tables p. 6

Incubation for further 10 min without CA abolished the neuronal fluorescence ( )

Incubation for further 10 min without CA had no apparent effect on the fluorescence intensity (2-3)

previously mentioned (Chap II B) the accumulation of CA was often confined to the peripheral part of slices of the nucleus caudatus putamen (Fig 7)

Incubation of slices of the vas deferens with L-dopa after MAO inhibition with nialamide (Table VI) resulted only in a weak neuronal fluorescence even after high concentrations of L-dopa which gave a high background fluorescence especially after 100 μg/ml. The neuronal fluorescence did not occur in the presence of the dopa decarboxylase inhibitor NSD 1015. Incubation of slices from the cerebral cortex with L-dopa in high concentrations (10-100 μg/ml) induced a general yellow green

TABLE VI Fluorescence intensity in nerve terminals after incubation of vas deferens slices with L-dopa. Rats pretreated with reserpine and mianserin were used

L-dopa concentration μg/ml		
100	+	(2)
10	+	(3) *
1	(+)	(1)
0.1	0	(4)

For symbols used see key to the tables p. 6

\* Increased background fluorescence

\* Addition of the dopa-decarboxylase inhibitor NSD 101a 50 μg/ml 15 min before L-dopa prevented fluorescence in nerve terminals (3)

background fluorescence. Under the present conditions no specific fluorescence was seen in nerve terminals.

In all areas of the brain an accumulation of CA in the endothelial walls was observed with a CA or L-dopa concentration of 1 μg/ml or above provided that the influence of MAO had been excluded (cf. Chap. III F).

## Discussion

There is strong evidence that the CA which accumulates under the present conditions after reserpine induced depletion of the endogenous CA is in fact localized to those nerve fibres that normally contain CA. The regional distribution of the fluorescent nerve terminals and non terminal axons after incubation with CA is exactly the same as that reported in untreated animals. No new nerve terminal systems were detected although incubation of slices from normal animals with CA often permitted more distinct localization of the terminal. Observations on slices from untreated rats also show unequivocally that it is only the nerve fibres with endogenous CA which have the ability to accumulate exogenous CA. This is further supported by the finding of the same selectivity in autonomic ganglion cells (Norberg, 1965) and of abolishment of the accumulation by sympathetic denervation (Hertting, Axelrod, Kopin and Whitby, 1961; Stromblad and Nickerson, 1961; Malmfors and Sachs, 1965).

The appearance of the nerve terminals and non terminal axons of both central and peripheral CA neurons in untreated rats and after accumulation is also indistinguishable although non terminal axons and terminals have about the same fluorescence intensity in the latter case. In whole mounts of rat iris, the varicosities were less pronounced after depletion with reserpine and accumulation with NA (Malmfors 1965). In sections of slices from the tissues used this is generally difficult to observe and distinct varicose fibres can be seen after reserpine pretreatment as well. In *in vitro* experiments on rat iris the same morphological appearance as *in vivo* was however observed (Hamberger and Malmfors 1967). The discrepancies in this respect between the tissues used and the iris are presumably to be ascribed to differences in the structure of these organs. In the nucleus caudatus putamen accumulation of CA is mainly confined to the peripheral parts of the slices probably due to the special structural organization of this area.

The occurrence of CA uptake after reserpine has earlier been reported both quantitatively (Lindmar and Muscholl 1964, Carlsson and Waldeck 1965, Carlsson 1966, Glowinski, Iversen and Axelrod 1966, Iversen 1967) and histochemically (Fuxe and Hilarp 1964, Hamberger, Malmfors, Norberg and Sachs 1964, Malmfors 1965, Norberg 1965, Fuxe and Ungerstedt 1966) as well as in isolated tissues (Hamberger and Mitsuoka 1965, Costi, Boulton, Hammer, Vogel and Brodie 1966, Haggendal and Hamberger 1967).

The experiments with varying incubation times showed that the accumulation as revealed by the fluorescence intensity reached a constant level after 20 min under the conditions used. Time curves made by use of labelled NA on slices from untreated animals suggest that equilibration is attained later (Dengler, Michaelson, Spiegel and Titus 1962, Ross and Renvy 1964). This difference may be due to quenching of the fluorescence (see Chap II B) especially after the high concentration and/or differences between untreated and reserpine pretreated animals. Furthermore the accumulation of CA in nerve terminals is detected histochemically whereas the total uptake i.e. both neuronal and extra neuronal is measured when using labelled compounds.

The experiments with varying concentrations showed a concentration dependent uptake in all tissues and of all drugs. The lowest

TABLE VII Effect of different incubation media, lowering of temperature and metabolic inhibitors on the accumulation *in vitro* of  $\alpha$  methyl NA in nerve terminals of central and peripheral CA neurons in reserpine pretreated rats

All experiments were compared with the accumulation induced by  $\alpha$  methyl NA 1 and 0.1 (cerebral cortex, hypothalamus, nucleus caudatus, putamen) or 0.03 (vas deferens)  $\mu\text{g/ml}$  in a Krebs Ringer (K R) bicarbonate medium gassed with  $\text{O}_2/\text{CO}_2$  and containing glucose (Composition: Na 143 mM, K 6.0 mM, Ca 1.3 mM,  $\text{Mg}^{2+}$  1.2 mM see also Chap. II A)

Modification of medium and/or addition of drugs	Inhibition in	
	Vas deferens nerve terminals	Central NA and NA nerve terminals
K R phosphate gassed with $\text{O}_2$ or $\text{N}_2$	None	(4)
K R bicarbonate gassed with $\text{N}_2/\text{CO}_2$	None	(4)
Glucose excluded	None or slight	(10)
K R bicarbonate gassed with $\text{N}_2/\text{CO}_2$ + exclusion of glucose	Marked	(4) Marked (2)
0.3 M Sucrose K R medium excluded	Complete	(3)
Incubation at 0°C in K R bicarbonate	Complete	(4) Complete (2)
2,4-Dinitrophenol $5 \cdot 10^{-3}\text{M}$ + exclusion of glucose	Marked	(?) Marked (?)
2,4-Dinitrophenol $10^{-3}\text{M}$ + exclusion of glucose	Marked or complete	(?) Marked (?)
Potassium cyanide $10^{-3}\text{M}$ + exclusion of glucose	Complete	(?)
Thiourea $10^{-3}\text{M}$	Slight or marked	(2) Marked (?)
K <sup>+</sup> exchanged for Na <sup>+</sup>	Complete	(4) Complete (2)
50% Na <sup>+</sup> exchanged for K <sup>+</sup>	Marked	(?) Marked (?)
Na <sup>+</sup> exchanged for La <sup>3+</sup>	Complete	(?)
50% Na <sup>+</sup> exchanged for La <sup>3+</sup>	Marked	(?)
Exclusion of $\text{Mg}^{2+}$ and $\text{Ca}^{2+}$	None	(?)

## Discussion

A number of modifications of the standard experimental set up have been found to interfere with the reserpine resistant accumulation of CA in central and peripheral CA nerve terminals. Most experiments were performed on *vis* deferens slices, and the main points investigated for the central CA terminals. The accumulation proved to be dependent on energy yielding processes, since it did not occur without oxygen and glucose and at a low temperature nor after addition of inhibitors of phosphorylating respiration such as dinitrophenol or cyanide. The experiments demonstrate that the energy required for CA accumulation can be generated by oxidation of either glucose or endogenous substrates. It is also evident that anaerobic glycolysis *per se* can provide sufficient amounts of high energy compounds (cf Whittam 1964).

Variations in ionic composition showed that a well balanced 'extracellular' ionic requirement was a prerequisite for CA accumulation. An absolute requirement appears to exist as no accumulation occurred after incubation with sucrose. Marked increases in potassium concentration which produced strong inhibition of accumulation caused depolarization of the cell membrane. This suggests that these two mechanisms may be correlated although it is also possible that other mechanisms than depolarization are involved (cf Gage and Quastel 1965, Hamberger and Malmfors 1967). On the other hand, cations—except for sodium and potassium—appeared to be of minor importance. The optimal sodium and potassium concentrations for CA accumulation resemble those stimulating the sodium/potassium activated ATPase of cell membranes (Kuroiwa, Sakamoto and Kato 1965). Analogous to the specific inhibitor of this enzyme ouabain (see e.g. Whittam 1964, Whittam and Blond 1964, Csaky 1965, Quastel 1965), depresses CA uptake.

Experiments on the uptake of radioactively labelled noradrenaline (Dengler, Michaelson, Spiegel and Titus 1962, Iversen and Kravitz 1966, Titus and Dengler 1966) and metamizol (Giachetti and Shore 1966, Ross and Renyi 1966) in isolated tissues of untreated animals have demonstrated inhibition of uptake under similar conditions. The present experiments have shown that the mechanism responsible for this uptake is insensitive to reserpine and is probably localized to the level of the cell membrane (for further discussion see Chap IV).

## E Effects of drugs on the uptake of catecholamines

### Results

The restitution of specific fluorescence by various catecholamines was described in Chap III, C. An account will now be given of the ability of certain drugs to prevent restitution. Special interest was focused on a comparison between the NA and DA nerve terminals and non terminal axons.

To investigate the possibility that reserpine affects the accumulation of CA after reserpine pretreatment, reserpine phosphate was added to the medium. In a concentration of  $10^{-6}$  M it did not affect the restitution *in vitro* of specific fluorescence in the vas deferens induced by  $\alpha$  methyl NA (1 and 0.03  $\mu$ g/ml).  $10^{-6}$  M reserpine was also used and did not influence restitution although a yellow background fluorescence of reserpine was observed.

Table VIII illustrates the ability of desipramine to prevent restitution with  $\alpha$  methyl NA. Under these conditions desipramine was active down to  $10^{-7}$  M on NA nerve terminals with  $\alpha$  methyl NA (0.03  $\mu$ g/ml), but the inhibition seemed to be dependent on the concentration of the amine. However neither the DA terminals in the nucleus caudatus putamen nor certain bundles of non terminal axons in the hypothalamic area were affected by desipramine (Figs 16-17) not even at a concentration of  $10^{-5}$  M, which depletes endogenous CA (Chap III, B). When DA was used after nortriptyline pretreatment instead of  $\alpha$  methyl NA (Table VIII) the same effect of desipramine was noted and the restitution in DA nerve terminal and non terminal axons was unfluenced.

Chlorpromazine also differed in its effect on NA and on DA nerve fibres (Table IX). In concentrations of  $10^{-6}$  and  $10^{-5}$  M in the incubation medium slight to marked prevention of restitution occurred in NA nerve terminals whereas the DA terminals were unaffected. With higher concentrations ( $10^{-4}$ — $10^{-3}$  M) however an effect was seen in the DA nerve fibres as well (+). Amphetamine and cocaine prevented restitution with  $\alpha$  methyl NA in DA and NA nerve fibres to the same degree (Table X). A marked effect was seen with (+) amphetamine  $0.5 \cdot 10^{-6}$  M and with cocaine  $10^{-6}$  M.

TABLE VIII Fluorescence intensity in NA and DA nerve terminals and non terminal axons of reserpine pretreated rats after incubation with  $\alpha$  methyl NA in the presence of desipramine<sup>1</sup>

Desipramine concentration	$\alpha$ methyl NA			
	1 $\mu$ g/ml		0.1 or 0.03 $\mu$ g/ml	
	NA nerve terminals and non terminal axons	DA nerve terminals and non terminal axons	NA nerve terminals and non terminal axons	DA nerve terminals and non terminal axons
10 <sup>-3</sup> M	0 (0)	+++ (2)	0 (2)	
10 <sup>-4</sup> M	0 (0)	+++ (4)	0 (0)	
10 <sup>-5</sup> M	(+) (4)	+++ (4)	0 (0)	++ (0)
10 <sup>-6</sup> M	+	+++ (1)	0 (3)	++ (-)
10 <sup>-7</sup> M	++ (2)		(+) (3)	++ (2)
10 <sup>-8</sup> M	++ (2)		+	(3)
0	++ (0)	+++ (4)	+	(0) ++ (0)
Nisalam le + DA				
	1 $\mu$ g/ml		0.1 $\mu$ g/ml	
	NA nerve terminals and non terminal axons	DA nerve terminals and non terminal axons	NA nerve terminals and non terminal axons	DA nerve terminals and non terminal axons
10 <sup>-3</sup> M	0 (0)	+++ (0)	0 (-)	++ (-)
0	++ (-)	+++ (0)	+	(-) ++ (2)

For symbols used see key to the tables p. 6

Results in vas deferens, cerebral cortex, hypothalamic area and nucleus caudatus putamen

TABLE IX Effect of chlorpromazine on the accumulation of  $\alpha$  methyl NA in NA and DA terminals and non terminal axons of reserpine pretreated rats

The effects were compared to the *in vitro* accumulation induced by  $\alpha$  methyl NA 1 and 0.1  $\mu$ g/ml

Chlorpromazine concentration	Inhibition in	
	NA nerve terminals and non terminal axons	DA nerve terminals and non terminal axons
10 <sup>-3</sup> M	Complete (0)	Complete (0)
10 <sup>-5</sup> M	Complete (3)	Slight to marked (3)
10 <sup>-7</sup> M	Marked (0)	None (0)
10 <sup>-9</sup> M	Slight to marked (-)	None (2)

<sup>1</sup> Results in vas deferens, cerebral cortex, hypothalamic area and nucleus caudatus putamen



TABLE X Effect of (+) amphetamine and cocaine on the accumulation of  $\alpha$ -methyl NA in NA and DA terminals and non terminal axons of reserpine pretreated rats

The effects were compared to the *in vitro* accumulation induced by  $\alpha$ -methyl NA 1 and 0.1  $\mu$ g/ml

(+) Amphetamine concentration	Inhibition in NA and DA nerve terminals and non terminal axons	
2 $\times$ 10 <sup>-4</sup> M—2.7 $\times$ 10 <sup>-3</sup> M	Complete	(3)
0.8 $\times$ 10 <sup>-3</sup> M	Marked	(3)
2 $\times$ 10 <sup>-4</sup> M	None or slight	(3)
0.8 $\times$ 10 <sup>-4</sup> M	None	(-)
Cocaine concentration		
10 <sup>-3</sup> M	Complete	(-)
10 <sup>-4</sup> M	Marked	(3)
10 <sup>-5</sup> M	Slight	(-)
10 <sup>-6</sup> M	None or light	(2)

<sup>1</sup> Results in vas deferens cerebral cortex hypothalamic area and nucleus caudatus putamen

<sup>2</sup> This concentration had an effect only on the terminals in the cerebral cortex

## Discussion

The drugs used in the present experiments are known to inhibit the accumulation of CA both in the peripheral (see *e.g.* Hertting, Axelrod and Whitby 1961; Axelrod 1964; Carlsson and Waldeck 1965; Malmfors 1966; Iversen 1967) and in the central nervous system (see *e.g.* Dengler, Spiegel and Titus 1961; Carlsson 1966; Carlsson, Fuxe, Hamberger and Lindqvist 1966a; Glowinski, Axelrod and Iversen 1966). The present experiments have shown that the *in vitro* uptake into the CA nerve terminals and non terminal axons is inhibited. Furthermore the data demonstrate differences between the accumulation in NA and DA neurons. Thus desipramine prevents accumulation in NA nerve terminals and non terminal axons only while those normally containing DA are unaffected even at a high concentration (10<sup>-3</sup> M) which depletes endogenous DA (Chap. III B) (see also Carlsson, Fuxe, Hamberger and Lindqvist 1966a; Fuxe, Hamberger and Malmfors 1966; Hamberger 1966; Häggendal and Hamberger 1967). The selective effect of desipramine provides a new possible

ity of differentiating between NA and DA neurons in the central nervous system (Fig. 17). The same inhibition pattern as that found with desipramine applies to other imipramine like drugs such as protriptyline (Carlsson Fuxe, Hamberger and Lindqvist 1966a). It is also evident from these results that the inhibitory effect of desipramine is dependent on the concentration of  $\alpha$  methyl NA. Thus a 10 to 30 fold increase in NA concentration requires a 100 fold increase in desipramine concentration to achieve the same inhibitory effect i.e. no accumulation can be detected (Table VIII). Studies with radioactive NA on uptake inhibition in cat heart slices showed an effect of desipramine down to  $10^{-7}$ M (Titus Matussek Spiegel and Brodie 1966).

(+) Amphetamine and cocaine prevent uptake in both NA and DA nerve fibres while chlorpromazine affects both NA and DA nerve terminals although the former at a lower concentration. For further discussion see Chap. IV.

## F Accumulation of catecholamines in the endothelial wall

### Results

Incubation of brain slices with CA ( $1 \mu\text{g/ml}$ ) resulted in intense specific fluorescence in the endothelial wall provided that the effect of MAO had been prevented (Table XI). The fluorescence was localized to cells in the endothelial wall (Figs 18 to 21) mainly pericytes although specific fluorescence was also visible in the endothelial cells especially after  $10 \mu\text{g/ml}$  (see Barkai and Anderson 1962; Landers, Chason, Gonzalez and Palutke 1962; Wolff 1963; Bertler, Falck, Owman and Rosengren 1966; Schwink and Wetzstein 1966). This type of fluorescent cell in the endothelial wall was not observed outside the central nervous system. In concentrations of 1 and  $10 \mu\text{g/ml}$  both DA and NA and their  $\alpha$  methylated analogues induced fluorescence while  $0.1 \mu\text{g/ml}$  produced none. Metaraminol (1 to  $10 \mu\text{g/ml}$ ) produced yellowish fluorescence in the cells in question.

Incubation with L-dopa also induced strong fluorescence in the endothelial wall (Table XII). The fluorescence did not occur if the tissue had been preincubated with the dopa decarboxylase inhibitor NSD 1015 ( $50 \mu\text{g/ml}$ ). Incubation with D-dopa also induced specific fluorescence although weaker than that of L-dopa.

TABLE VI Fluorescence intensity in the endothelial walls of cerebral capillaries after incubation of cerebral cortex slices with CA or metaraminol

Drug	Concentration $\mu\text{g/ml}$	
NA	10	0 (2)
	1	0 (3)
	0.1	0 (20)
$\alpha$ Methyl NA or niplamide + NA	10	+ (8)
	1	+ (10)
	0.1	0 (20)
$\alpha$ Methyl DA or niplamide + DA	10	+ (1)
	1	+ (15)
	0.1	0 (4)
Metaraminol	10	+ (3)
	1	(+) (3)
	0.1	0 (3)

For symbols used see key to the tables p 6

<sup>1</sup> Rats pretreated with reserpine

Incubation for further 15 min without CA had no apparent effect on the fluorescence intensity (2)

TABLE VII Fluorescence intensity in the endothelial walls of cerebral capillaries after incubation of cerebral cortex slices<sup>1</sup> with dopa

L-dopa concentration $\mu\text{g/ml}$		D-dopa concentration $\mu\text{g/ml}$			
100	+	(2)	100	+	(2)
10	+	(3)	10	0	(2)
1	+	(4)	1	0	( )
0.1	0	(1)			

For symbols used see key to the tables p 6

Rats pretreated with reserpine and niplamide

Addition of the dopa-decarboxylase inhibitor NSD 1015 50  $\mu\text{g/ml}$  15 min before L-dopa prevented fluorescence in the endothelial walls (3)

The effect of modification of media and addition of drugs was also investigated. Exclusion of oxygen and glucose or incubation at a low temperature or addition of dinitrophenol in combination with exclusion of glucose all affected the occurrence of fluorescence

TABLE VIII Fluorescence intensity in the endothelial walls of cerebral capillaries after incubation of cerebral cortex slices<sup>1</sup> with a methyl NA in the presence of drugs  
a Methyl NA 1  $\mu\text{g/ml}$  was used in all experiments

Drug	Concentration		
Desipramine	$10^{-8}$ — $10^{-6}\text{M}$	0	(4)
	$10^{-8}$ — $10^{-6}\text{M}$	(+)	(7)
	$10^{-8}$ — $10^{-6}\text{M}$	+	(3)
(+)-Amphetamine	$2.7 \cdot 10^{-6}\text{M}$	0	(2)
	$0.8 \cdot 10^{-6}\text{M}$	(+)	(1)
	$2.7 \cdot 10^{-6}\text{M}$	+	(2)
Cocaine	$10^{-8}$ — $10^{-6}\text{M}$	0	(2)
	$10^{-8}$ — $10^{-6}\text{M}$	+	(3)
No drug		+	(12)

For symbols used see key to the tables p 8

Rats pretreated with reserpine

similarly to the effects in central nerve terminals (see Table VII) Desipramine (+)-amphetamine and cocaine prevented the occurrence of specific fluorescence in the endothelial wall (Table VIII)

### Discussion

Cells in the endothelial wall of capillaries in the central nervous system accumulated CA *in vitro* when incubated in concentrations of 1  $\mu\text{g/ml}$  or higher provided that the influence of MAO had been eliminated (see also Hamberger and Masuoka 1963) No differences were found between untreated (Chap III A) and reserpine pretreated rats The mechanism underlying this accumulation in central capillaries is presumably specific since inhibition of energy yielding processes prevented accumulation The concentration ability is however far below that of the nerve terminals and non terminal axons Cells in the endothelial wall were also observed to accumulate metaraminol since it can be visualized extraneuronally due to fluorescence with an emission peak at 310 m $\mu$  (Sachs 1963 Johnson and Ritzen 1966)

Fluorescence after incubation with L dopa was found with about

the same concentrations as those of CA. As the dopa decarboxylase inhibitor NSD 1015 prevented fluorescence it is likely that the fluorescence is due to a CA probably DA, and that L dopa itself cannot accumulate. The same observations have earlier been made *in vivo* (Bertler, Falck and Rosengren 1963, Bertler, Falck, Owman and Rosengren 1966). The fact that D dopa induces less intense fluorescence than L dopa may be due to the specificity of the dopa decarboxylase.

It is interesting to note that drugs which block the reserpine resistant accumulation in nerve terminals such as desipramine, cocaine and (+) amphetamine are also able to block the extra-neuronal accumulation. The drug concentration for blocking these two types of accumulation is about the same which must be borne in mind when evaluating biochemical measurements on *ex vivo* accumulation of CA and its inhibition (cf Haggendal and Hamberger 1967).

It is tempting to assume that the cells in the endothelial wall play a role in the transfer of CA between blood and brain and thus take part in the blood brain barrier for CA. No fluorescence is found even after very high *in vivo* doses of CA (Bertler, Falck and Rosengren 1963, Bertler, Falck, Owman and Rosengren 1966). After mild chemically induced blood brain barrier damage however CA administered *in vivo* can accumulate in these cells (Hamberger and Hamberger 1966, Flodmark, Hamberger, Hamberger and Steinwall to be publ.).

## GENERAL DISCUSSION

In most studies, the uptake of CA has been detected by measuring the accumulation in whole organs or parts of them. As pointed out by Iversen (1965 b) the distinction between uptake and accumulation is important in connection with CA. Uptake refers to the passage of a substance from extra- to intracellular space but is not primarily related to its storage, which also means that accumulation must include uptake. Injection of labelled NA into an untreated animal results in an uptake and accumulation of the injected amine (see e.g. Axelrod 1964 Iversen 1965 b 1967). After reserpine pretreatment no accumulation of NA can be detected although the uptake is unaffected (Lindmar and Muscholl 1964 Iversen Glowinski and Axelrod 1965). Administration of an MAO inhibitor however permits detection of an accumulation of the CA (Tuxe and Hillarp 1964 Hamberger Malmfors Norberg and Sachs 1964 Carlsson 1965 Carlsson and Wuldeck 1965 Malmfors 1965 Norberg 1965 Carlsson 1966). There is now strong evidence that reserpine neither *in vivo* after different times (Carlsson 1965 Dahlstrom Tuxe and Hillarp 1965 Malmfors 1965) nor *in vitro* affects this uptake.

After incubation of vas deferens slices from reserpine pretreated rats with  $\alpha$  methyl NA (0.003–0.01  $\mu\text{g/ml}$ ) weak specific fluorescence occurs in the nerve terminals. From model experiments (Ritzen 1966) the average intraneuronal NA content must be assumed to be about 10  $\mu\text{g/g}$  wet weight in this case as the relative fluorescence yield of  $\alpha$  methyl NA and NA is the same (Jonsson 1967 a). When comparing the concentration of CA outside the nerve terminal and the average concentration inside a difference of at least 1000 times is found. These calculations are naturally unsafe but receive support from earlier calculations (Hamberger and Masuoka 1965).

The reserpine resistant uptake was characterized in Chap. III D. The process was found to be energy dependent since the addition of dinitrophenol or cyanide inhibited the accumulation.

Incubation at 0°C or in the absence of oxygen and glucose markedly suppressed the uptake. The membrane ATPase inhibitor ouabain also prevented accumulation when used in a high concentration. The experiments on the ionic composition of the medium showed that a certain concentration of sodium and potassium ions was necessary for uptake of CA. As pointed out in Chap. III C, experiments on prevention of uptake of NA (Dengler, Michaelson, Spiegel and Titus 1962, Iversen and Kravitz 1966, Titus and Dengler 1966) or metaraminol (Girichetti and Shore 1966, Ross and Renyi 1966) have previously been made with e.g. metabolic inhibitors and ouabain and the results are supported by the present ones. In those studies it is apparent that the initial uptake was dependent on the reserpine resistant mechanism even if the uptake and storage in amine storage granules were intact (cf. Lindmar and Muncholl 1964) which implies that the same mechanism was studied as in the present study. When taking all these facts into consideration there is thus little doubt that the reserpine resistant uptake of CA fulfills the demands of an active carrier mediated transport (see Wilbrandt and Rosenberg 1961, Schanler 1962, Csaky 1963).

The reserpine resistant uptake mechanism of catecholamines has been shown to exist in the whole CA neuron i.e. cell body, non terminal axon and terminal (Fuxe and Hillarp 1964, Hamberger, Malmfors, Norberg and Sachs 1964, Malmfors 1965, Norberg 1965, Fuxe and Ungerstedt 1966) despite the varying content of amine storage granules (see the following). This favours the view that it is localized to the level of the cell membrane. This view is also borne out by pharmacological experiments. Desipramine is known to inhibit the reserpine resistant uptake in NA neurons (cf. Chap. III D) but does not affect the uptake into amine storage granules except in much higher concentrations (Carlsson, Hillarp and Waldeck 1963). In animals depleted of their endogenous CA with an inhibitor of the CA synthesis which does not interfere with the  $Mg^{++}$  ATP dependent storage in the amine granules, desipramine prevents exogenous CA from entering the NA nerve terminals and reaching the uptake storage sites (Malmfors, pers. comm.).

That the amine storage granules are the main site of the reserpine resistant uptake is further contradicted by the finding that after reserpine resistant accumulation of NA non terminal axons

and terminals have a fluorescence of similar intensity despite a low and high content of amine storage granules respectively (Richardson 1964; Hokfelt 1967). These findings make it highly probable that most of the CA accumulated after reserpine pretreatment is localized outside the amine storage granules and more or less free in the cytoplasm. It nevertheless seems possible that the CA which accumulates after reserpine pretreatment is partly bound to an intraneuronal structure. It has been shown that isolated amine storage granules can take up CA also after reserpine treatment provided that the CA concentration in the incubation medium is high (Stjärne 1964; Euler and Lishajko 1965). It is highly probable that a high intraneuronal concentration can occur under the present experimental conditions. In studies of the subcellular distribution of NA taken up after reserpine a certain amount of NA was found in the amine storage granule fraction (Lundborg and Stitzel 1967; Stitzel and Lundborg 1967). Histochemical experiments have suggested that part of the exogenous CA after reserpine is localized to intraneuronal structures with the same distribution as those normally containing NA, i.e. the amine storage granules (Hamberger and Malmfors 1967). The mechanism responsible for this supposed binding must however be independent of the  $Mg^{++}$  ATP dependent mechanism in amine storage granules.

The high concentrations of CA (10–1  $\mu$ g/ml) used in the present study are considerably higher than those used in studies with radioactive NA (Dengler, Michaelson, Spiegel and Titus 1962; Titus, Mitussek, Spiegel and Brodie 1966). When using these high concentrations the authors found the same CA concentration in both slice and medium. It is however evident from the present results that a large concentration difference certainly exists inside and outside the nerve terminals even with high CA concentrations in the incubation medium. The failure of earlier studies to reveal an uptake of CA at high concentrations may be due to the greater amount of CA being present extraneuronally, and masking the specific uptake (cf. Haggendal and Hamberger 1967). As the CA present intraneuronally more easily diffuses out of the slice if the amines are removed from the incubation medium, careful rinsing of the slices after incubation with radioactive CA would in all probability



di close accumulation of isotope even with high CA concentrations in the medium

The CA concentrations used in the present experiments are probably comparable to those normally present just outside the cell membrane of a varicosity when transmitter is released by nerve impulses. It may be justified to make some calculations of this matter. One varicosity in the rat iris or vas deferens contains about  $5 \cdot 10^{-3}$  pg NA (Dahlstrom Haggendal and Hokfelt 1966). Depletion of endogenous CA can be achieved by nerve impulses and about 30 000 impulses are needed for this purpose provided that CA synthesis has been inhibited (Malmfors pers comm). Kernell and Sedvall (1964) reported that in the cat 50 000 impulses are needed to give a 55 to 70 per cent reduction in the endogenous NA with synthesis and re uptake intact. Assuming from these data that 10 000 to 50 000 impulses are needed to deplete endogenous NA if no re uptake or synthesis occurs, that the varicosity is a sphere about  $1\text{--}1.5 \mu$  in diameter and that the transmitter released is evenly distributed in a 200–300 Å thick layer (cf Taxi 1965) around the whole varicosity, one nerve impulse should liberate about  $1\text{--}5 \cdot 10^{-7}$  pg NA and when no uptake occurs the concentration of CA just outside the varicosity should be of the order of 0.5–8  $\mu\text{g/ml}$ . This suggests that the concentrations used in the present study may well be compatible with those occurring locally under physiological conditions.

In experiments on isolated perfused rat hearts Iversen (1965a, b) found a new catecholamine uptake process (Uptake<sub>2</sub>) which was detectable with high NA concentrations (1–40  $\mu\text{g/ml}$ ) in the perfusion medium. The properties of Uptake<sub>2</sub> differed from those of Uptake<sub>1</sub>, which operated at low perfusion concentrations of NA (below 1  $\mu\text{g/ml}$ ) and showed similarities to the uptake discussed in the present paper. It was suggested that both Uptake<sub>1</sub> and Uptake<sub>2</sub> were localized to the adrenergic nerves (Iversen 1965, b). However histochemical experiments recently carried out in our laboratory have provided strong evidence that most of the CA taken up by Uptake<sub>2</sub> is localized extraneuronally to cells in the connective tissue and probably to the muscle cells (Malmfors pers comm). The suggestion that Uptake<sub>2</sub> is related to the uptake in the non terminal axons and cell bodies (Iversen 1965, b) is strongly contradicted by the finding that the w

adrenergic neuron reacts uniformly on treatment with drugs (Malmfors 1965 Norberg 1965)

The inhibition of uptake by drugs has shown that, in all probability differences exist in the reserpine resistant accumulation of CA in NA and DA neurons. While desipramine inhibited uptake in NA neurons, no effect at all could be found in DA neurons not even in very high concentrations. Chlorpromazine inhibited uptake in both NA and DA nerve terminals although higher concentrations were needed for an effect on the latter. Cocaine and (+) amphetamine on the other hand, prevented uptake in NA and DA nerve terminals and non terminal axons to the same extent (cf Haggendal and Hamberger 1967). In connexion with (+) amphetamine a releasing activity is probably involved in the prevention of accumulation of CA (Carlsson and Waldeck 1966, Carlsson, Fuxe, Hamberger and Lindqvist 1966a, Hamberger and Malmfors 1967). At least with high (+) amphetamine concentrations, an inhibition of uptake is likely to have occurred since no intraneuronal amine could be detected although the tissue was frozen directly after incubation with CA. The same inhibition pattern in DA nerve terminals has been obtained *in vivo* (Fuxe, Hamberger and Malmfors 1966) and by use of intracerebral injections (Fuxe and Ungerstedt pers comm). Intracerebral injections of labelled NA gave almost similar results although they were not interpreted in this way (Glowinski, Axelrod and Iversen 1966). On the basis of the present pharmacological data it is thus highly probable that the mechanisms for reserpine resistant accumulation in DA and NA nerve fibres differ. The nature of this difference remains to be solved. The present experiments have also provided strong evidence that NA nerve fibres in the central and peripheral nervous system behave similarly in isolated tissues with respect to uptake and accumulation of CA and its prevention by drugs.

The *in vitro* experiments performed thus show that central CA nerve terminals have a highly efficient mechanism for accumulating CA as earlier known for the sympathetic adrenergic neuron. This mechanism is presumably located to the level of the cell membrane, is insensitive to reserpine, and fulfills the demands of a carrier mediated transport. It seems probable that this mechanism constitutes one of the most important mechanisms for inactivation of the released transmitter (Axelrod 1964, Hattori, Hürthmann

and Thoenen 1964 Iversen 1967) Furthermore this mechanism appears to be of great importance in psychopharmacology, as certain drugs known to inhibit the uptake of CA are effective antidepressants (Carlsson, Fuxe Hamberger and Lindqvist 1966 a see Garattini and Dukes 1967) This implies that the present technique can be used in a search for new antidepressant drugs

## SUMMARY

The uptake and accumulation of CA have been studied in isolated tissues mainly from reserpine pretreated rats. After incubation of thin slices of the brain and vas deferens with dopa or CA in a Krebs Ringer bicarbonate buffer the slices were investigated by the histochemical fluorescence method of Falck and Hillarp. This method permits the visualization of dopa and CA as NA and DA. In the present work the basis for standardization of the formaldehyde gas treatment which is a crucial step in the technique is also described.

The experiments performed *in vitro* show that NA and DA terminals and non terminal axons in the brain have a reserpine resistant mechanism for accumulating CA as earlier known to apply to the peripheral adrenergic neuron. This mechanism which is probably localized to the level of the cell membrane is shown to have a high concentration ability, to be dependent on energy yielding processes and to require a certain extracellular concentration of sodium and potassium. It is postulated that the reserpine resistant accumulation of CA is an active carrier mediated transport.

The inhibition of CA accumulation by drugs was studied with special interest focused on a comparison between NA and DA neurons. Whereas (+) amphetamine and cocaine prevented accumulation of CA in both NA and DA terminals and non terminal axons to about the same extent, desipramine inhibited uptake only in those normally containing NA. Desipramine is found to have no effect on DA terminals and non terminal axons not even in very high concentrations which deplete endogenous NA or DA within 15 min.

Apart from the described uptake and accumulation in nervous elements it is found that cells in the endothelial wall pericytes and possibly also endothelial cells in the brain capillaries accumulate CA after incubation with dopa or CA provided that MAO is inhibited. This accumulation was energy dependent and could be prevented by drugs, such as desipramine, (+) amphetamine and cocaine and it is suggested that these cells play a role in the blood brain barrier for CA.

## ACKNOWLEDGEMENTS

For generous supplies of drugs I am indebted to the following companies: Swedish Ciba Stockholm Sweden (Serpasil®) The Swedish Pfizer Stockholm Sweden (Naramid®) Hoechst Anilin AB Goteborg Sweden (Corbisil®) Hassle Goteborg Sweden (α methyl DA) Merck Sharp and Dohme Research Lab Rahway N Y U.S.A. through Firma Lindblom and Co Stockholm Sweden (Arimine®) Geigy Basel Switzerland (Pertofran®) Leo Helsingborg, Sweden (Hibernal®) and Smith and Nephew Research Ltd England (NSD 1015). Many thanks are also due to Gillette AB Stockholm Sweden for generous gifts of Valet 3 blade Strips. The skillful technical assistance of Miss Annika Degervall and Mrs Ulla Hylger is gratefully acknowledged. The investigation was supported by a grant from the Swedish Medical Research Council (B67—12\—711—02).

## REFERENCES

- ANDÉN N E K FUXE B HAMBERGER and T HÖKFELT A quantitative study on the nigro neostriatal dopamine neuron system in the rat *Acta physiol scand* 1966 67 306—312
- ANDÉN N E K FUXE and T HÖKFELT Effect of some drugs on central monoamine nerve terminals lacking nerve impulse flow *European J Pharmacol* 1967 (in press)
- ANDÉN N E K FUXE and U UNGERSTEDT Monoamine pathways to the cerebral cortex and the cerebellum *Experimentia* 1967 (in press)
- ANGELAKOS E T and M KING Histochemical demonstration of catecholamine uptake by adrenergic fibers *J Histochem Cytochem* 1965 13 282—285
- AXELROD J The uptake and release of catecholamines and the effect of drugs In *Progr Brain Res* (ed H E HAWKICH and W A HAWKICH) Elsevier Publ Co Amsterdam 1961 8 81—89
- AXELROD J E GORDON G HERTTING I J KOPIN and L T POTTER On the mechanism of tachyphylaxis to tyramine in the isolated rat heart *Brit J Pharmacol* 1962 19 56—63
- BARNA T and P J ANDERSON Histochemical methods for acid phosphatase using hexazotium pararosanilin as coupler *J Histochem Cytochem* 1962 10 741—753
- BERTLER A B FALCK C OWMAN and E ROSENGREN The localization of monoaminergic blood brain barrier mechanisms *Pharmacol Rev* 1966 18 369—386
- BERTLER A B FALCK and E ROSENGREN The direct demonstration of a barrier mechanism in the brain capillaries *Acta pharmacol* 1968 20 317—321
- CARLSSON A Drugs which block the storage of 5 hydroxytryptamine and related amines In *Handbuch der Exp Pharmacol* (ed V ERSKAMER) Springer Verlag Berlin Göttingen Heidelberg 1965 19 529—592
- Pharmacological depletion of catecholamine stores *Pharmacol Rev* 1966 18 511—549
- CARLSSON A B FALCK and N Å HILLARP Cellular localization of brain monoamines *Acta physiol scand* 1962 56 Suppl 196 1—28
- CARLSSON A K FUXE B HAMBERGER and M LINDQVIST Biochemical and histochemical studies on the effects of amphetamine like drugs and (+) amphetamine on central and peripheral catecholamine neurons *Acta physiol scand* 1966a 67 481—497
- The effect of (+) amphetamine on various central and peripheral catecholamine containing neurons *J Pharm Pharmacol* 1966b 18 128—130
- CARLSSON A N Å HILLARP and B WALDFCK Analysis of the  $Mg^{++}$  ATP dependent storage mechanism in the amine granules of the adrenal medulla *Acta physiol scand* 1963 59 Suppl 215 1—28

## ACKNOWLEDGEMENTS

For generous supplies of drugs I am indebted to the following companies: Swedish Ciba, Stockholm Sweden (Serpasil®), The Swedish Pfizer, Stockholm Sweden (Nimid®), Hoechst Aktien AB Göteborg Sweden (Corbasil®), Hassle Göteborg Sweden (α-methyl DA), Merck Sharp and Dohme Research Lab Rahway N.Y. U.S.A. through Firma Lindblom and Co Stockholm Sweden (Arimine®), Geigy, Basel Switzerland (Perlofrin®), Ico Hälsingborg Sweden (Hibernal®) and Smith and Nephew Research Ltd, England (NSD 1015). Many thanks are also due to Gillette AB Stockholm Sweden for generous gifts of Valet blade strips. The skillful technical assistance of Miss Annika Degervall and Mrs Ulla Flyger is gratefully acknowledged. The investigation was supported by a grant from the Swedish Medical Research Council (B67—12\—711—02).

# REFERENCES

- ANDERSON F K, FULF B, HAMBERGER and J P 1961  
study on the nigro-neostriatal dopamine neuron system  
*physiol scand* 1966 67 306-312
- ANDERSON F K, FULF and T HOKFELT Effect of  
monoamine nerve terminals lacking nerve impulses  
*Pharmacol* 1967 (in press)
- ANDERSON F K, FULF and U UNGERSTEDT  
the cerebral cortex and the cerebellum *Experientia* 1967 23 1-10
- ANGLAKOS E I and M KING Histochemical demonstration of  
cholinergic uptake by adrenergic fibers *J Histochem Cytochem* 1967 16 282-283
- AXELROD J The uptake and release of catecholamines and related  
drugs In *Progr Brain Res* (ed H F HAWKINS and J P WICK)  
Elsevier Publ Co Amsterdam 1961 8 81-103
- AXELROD J, L GORDON, C HERTTING, I J KOPPEL and L T PERKINS  
the mechanism of tachyphylaxis to tyramine in the rat  
*Brit J Pharmacol* 1962 19 56-67
- BARRA T and P J ANDERSON Histochemical methods for the demonstration of  
phosphatase using hexazonium pararosaniline as coupler *J Histochem Cytochem* 1962 10 741-753
- BERTLER A, B FALCK, C OLMAY and E ROSENBERG The localization of  
of monoaminergic blood brain barrier mechanisms *Pharmacol* 1966 18 369-386
- BERTLER A, B FALCK and E ROSENBERG The direct demonstration of  
barrier mechanism in the brain capillaries *Acta pharmacol* 1967 18 317-321
- CARLSSON A, Drugs which block the storage of 5 hydroxytryptamine and  
related amines In *Handbuch der Exp Pharmacol* (ed V ERLANGER)  
Springer Verlag Berlin Göttingen Heidelberg 1963 19 520-539
- Pharmacological depletion of catecholamine stores *Pharmacol Rev*  
1966 18 511-519
- CARLSSON A, B FALCK and N A HILLARP Cellular localization of brain  
monoamines *Acta physiol scand* 1962 66 Suppl 196 1-28
- CARLSSON A, K FOXE, B HAMBERGER and M LINDQVIST Biochemical  
and histochemical studies on the effects of imipramine like drugs and  
(+) amphetamine on central and peripheral catecholamine neurons  
*Acta physiol scand* 1966a 67 481-497
- The effect of (+) amphetamine on various central and peripheral catecholamine-containing neurons *J Pharm Pharmacol* 1966b 18 129-130
- CARLSSON A, N A HILLARP and H WALBECK Analysis of the Mg<sup>2+</sup>  
ATP dependent storage mechanism in the amine granules of the  
adrenal medulla *Acta physiol scand* 1963 69 Suppl 215 1-38



- CARLSSON A and M LINDQVIST *In vivo* decarboxylation of a methyl dopa and a methylmetatyrosine *Acta physiol scand* 1962 54 87—91
- CARLSSON A and B WALDECK Mechanism of amine transport in the cell membranes of the adrenergic nerves *Acta pharmacol* 1963 22 293—300
- Effects of amphetamine tyramine and protriptyline on reserpine resistant amine-concentrating mechanisms of adrenergic nerves *J Pharm Pharmacol* 1966 18 232—233
- CASPERSON T A A HILLARI and M RITZEN Fluorescence microspectrophotometry of cellular catecholamines and 5 hydroxytryptamine *Exp Cell Res* 1966 4 415—428
- COMBODI H and A A HILLARI Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 1 Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 67 Dimethoxyisochinolinderivaten und Formaldehyd *Helv chim Acta* 1963 46 2135—2139
- Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 2 Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd *Helv chim Acta* 1964 47 911—918
- COMBODI H A A HILLARI and G JOHNSON Fluorescence methods for the histochemical demonstration of monoamines 3 Sodium borohydride reduction of the fluorescent compounds as a specificity test *J Histochem Cytochem* 1964 12 582—586
- COMBODI H and G JOHNSON Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 5 Identifizierung des fluoreszierenden Produktes aus Modellversuchen mit 5 Methoxytryptamin und Formaldehyd *Acta histochem* 1965 22 247—258
- Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen 6 Identifizierung der fluoreszierenden Produkte aus m-Hydroxyphenylethylaminen und Formaldehyd *Helv chim Acta* 1966 49 798—806
- The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines A review on the methodology *J Histochem Cytochem* 1967 15 65—78
- COMBODI H G JOHNSON and T MALMFORSS Factors affecting the quality and intensity of the fluorescence in the histochemical method of demonstration of catecholamines *Acta histochem* 1969 26 267—300
- COSTA E D J BOLLIN W HAMMILL W VOGEL and B B BRODIE Interactions of drugs with adrenergic neurons *Pharmacol Rev* 1966 18 57—108
- CRAIG T J Transport through biological membranes *Ann Rev Physiol* 1963 25 115—130
- DANIELSSON A and K LUXE Evidence for the existence of monoamine containing neurons in the central nervous system I Demonstration of monoamines in the cell bodies of brain stem neurons *Acta physiol scand* 1964 12 Suppl 27 1—33

- Evidence for the existence of monoamine neurons in the central nervous system II Experimentally induced changes in the intraneuronal amine levels of bulbo spinal neuron system *Acta physiol scand* 1965 64 Suppl 217 1—36
- DAHLSTROM A K IUNE and N A HILLARI Site of action of reserpine *Acta pharmacol* 1965 22 277—292
- DAHLSTROM A and J HAGGENDAL Recovery of noradrenaline levels after reserpine compared with the life span of amine storage granules in rat and rabbit *J Pharm Pharmacol* 1966 18 750—752
- DAHLSTROM A J HAGGENDAL and T HOKFELT The noradrenaline content of the varicosities of sympathetic adrenergic nerve terminals in the rat *Acta physiol scand* 1966 67 289—294
- DENGLER H J I A MICHAELSON H E SPIEGEL and E TITUS The uptake of labeled norepinephrine by isolated brain and other tissues of the cat *Int J Neuropharmacol* 1962 1 23—38
- DENGLER H J H E SPIEGEL and E TITUS Effects of drugs on uptake of isotopic norepinephrine by cat tissues *Nature* 1961 191 816—817
- FULFR U S v Noradrenaline Ch C Thomas Springfield Ill 1956
- Catecholamines in nerve and organ granules In *Mechanisms of release of biogenic amines* (ed U S v LULFR S ROSELL and H UYNAS) Pergamon Press 1966 211—222
- LULFR U S v and F LUBJAKO Catecholamine release and uptake in isolated adrenergic nerve granules *Acta physiol scand* 1963 57 468—480
- Effects of drugs on the storage granules of adrenergic nerves In *Pharmacology of cholinergic and adrenergic transmission* (ed G B KOELLE W W DOUGLAS and A CARLSSON) Proc Second Intern Pharmacol Meeting Pergamon Press 1965 3 215—259
- FALCK B Observations on the possibilities of the cellular localization of monoamines by a fluorescence method *Acta physiol scand* 1962 56 Suppl 197 1—25
- FALCK B N A HILLARP G THIEME and A TORP Fluorescence of catecholamines and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962 10 348—354
- FALCK B and C OWMAN A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines *Acta Univ Lund* 1965 Section II No 7 1—24
- FIELD J Respiration of tissue slices *Metb Med Res* 1948 1 269—307
- FISCHER K Neues Verfahren zur massanalytischen Bestimmung des Wassergehaltes von Flüssigkeiten und festen Körpern *Angew Chemie* 1935 48 394—396
- FUXE K Cellular localization of monoamines in the median eminence and infundibular stem of some mammals *Z Zellforsch* 1961 61 710—721

- FELT K Evidence for the existence of monoamine neurons in the central nervous system III The monoamine nerve terminal *Zellforsch* 1965a 6: 573—596
- Evidence for the existence of monoamine neurons in the central nervous system IV The distribution of monoamine nerve terminals in the central nervous system *Acta physiol scand* 1965b 64 Suppl 247 39—83
- FELT K, B HAMMEGER and T MALMFORSS Inhibition of amine uptake in tubero-infundibular dopamine neurons and in catecholamine cell bodies of the area postrema *J Pharm Pharmacol* 1966 18 513—514
- FELT K and N A HILLARP Uptake of L-dopa and noradrenaline by central catecholamine neurons *Life Sci* 1964 3 1403—1406
- FELT K, T HOKFELT and O NILSSON A fluorescence and electronmicroscopic study on certain brain regions rich in monoamine terminals *Amer J Anat* 1965 177 33—46
- FELT K and U UNGERSTEDT Localization of catecholamine uptake in rat brain after intraventricular injection *Life Sci* 1966 5 1817—1821
- GARR P W and D M J QUASTEL Dual effect of potassium on transmitter release *Nature* 1965 206 625—626
- GRANTING S and M N C DICKES (Editors) Antidepressant drugs Excerpta Medica Amsterdam 1967
- GRISCHETTI A and I A SNOW Studies *in vitro* of amine uptake mechanisms in heart *Biochem Pharmacol* 1966 1, 607—614
- GLOWINSKI J, J AXELROD and I L IVERSEN Regional studies of catecholamines in the rat brain IV Effects of drugs on the disposition and metabolism of H<sup>3</sup> norepinephrine and H<sup>3</sup> dopamine *J Pharmacol exp Ther* 1966 153 30—47
- GLOWINSKI J, I L IVERSEN and J AXELROD Storage and synthesis of norepinephrine in the reserpine treated rat brain *J Pharmacol exp Ther* 1966 157 385—399
- HAMMELT W, A HUBMANN and H THOENEN Scheinbar paradoxe Beeinflussung von peripheren Noradrenalinwirkungen durch einige Thyreolytika *Helv Physiol Pharmacol Acta* 1964 22 15—33
- HACCARDAL J and B HAMMEGER Quantitative *in vitro* studies on noradrenaline uptake and its inhibition by amphetamine, desipramine and chlorpromazine *Acta physiol scand* 1967 (in press)
- HAMMEGER A and B HAMMEGER Uptake of catecholamines and penetration of trypsin blue after blood brain barrier lesions *Z Zellforsch* 1966 70 386—392
- HAMMEGER B Uptake of catecholamines in rat brain slices and its inhibition by drugs Abstr VII Scand Congr of Physiology Abstr *Acta physiol scand* 1966 68 Suppl 27 69
- HAMMEGER B and T MALMFORSS Uptake and release of a methyl noradrenaline after reserpine pretreatment A histochemical study *Acta physiol scand* 1967 (in press)

- HAMBERGER H, T MALMFOUR, K A NORBERG and C SACHS Uptake and accumulation of catecholamines in peripheral adrenergic neurons of reserpinized animals studied with a histochemical method *Biochem Pharmacol* 1961 13 811—811
- HAMBERGER H, T MALMFOUR and C SACHS Standardization of paraformaldehyde and of certain procedures for the histochemical demonstration of catecholamines *J Histochem Cytochem* 1961 13 147
- HAMBERGER H and D MASLOKA Localization of catecholamine uptake in rat brain slices *Acta pharmacol* 1965 22 363—368
- HAMBERGER H, K A NORBERG and I OLSSON Intraneuronal binding of catecholamines and 3,4-dihydroxyphenylalanine (dopa) in salivary glands *Acta physiol scand* 1967 69 1—12
- HAYCOCK R P, P B SHETH and W J MAYER Spectrophotofluorometry of reserpine, other rauwolfia alkaloids and related compounds *J Amer pharm Sci* 1959 48 179—185
- HERTTING G, J AXELROD, I J KOTIN and I G WHITBY Lack of uptake of catecholamines after chronic denervation of sympathetic nerves *Nature* 1961 189 66
- HERTTING G, J AXELROD and I G WHITBY Effect of drugs on the uptake and metabolism of H<sup>3</sup> norepinephrine *J Pharmacol exp Ther* 1961 134 146—153
- HILLARP N A, K FUXE and A DAHLSTROM Central monoamine neurons. In *Mechanisms of release of biogenic amines* (ed U S V ELIER, S ROSEFEL and H ULLMANN) Pergamon Press, 1966a 31—37
- Demonstration and mapping out of central neurons containing dopamine, noradrenaline and 5-hydroxytryptamine and their reactions to psychopharmaca *Pharmacol Rev* 1966b 18 727—741
- HOKFELT T Ultrastructural studies on adrenergic nerve terminals in the albino rat brain after pharmacological and experimental treatment *Acta physiol scand* 1967 69 125—126
- IJERSEN L L The uptake of catecholamines at high perfusion concentrations in the rat isolated heart. A novel catecholamine uptake process *Brit J Pharmacol* 1965a 20 18—33
- The inhibition of noradrenaline uptake by drugs. In *Advances in drug research* (ed N J HARPER and A B SIVINOVOS) 1965b 2 1—46
- *The uptake and storage of noradrenaline in sympathetic nerves* Univ Press Cambridge 1967
- IJERSEN L L, J GLOWINSKI and J AXELROD The uptake and storage of H<sup>3</sup> norepinephrine in the reserpine pretreated rat heart *J Pharmacol exp Ther* 1965 150 173—183
- IJERSEN L L and E A KRAVITZ Sodium dependence of transmitter uptake at adrenergic nerve terminal *Mol Pharmacol* 1966 2 360—362
- JONSSON C Fluorescence studies on some 6,7-substituted 3,4-dihydroxyquinolines formed from 3-hydroxytyramine (dopamine) and formaldehyde *Acta chem scand* 1966 20 2755—2762



- MUROI K and K OGURA The determination of the water content of the low polymers of formaldehyde by the Karl Fischer Method. I The determination of the adsorbed water and bound water formed by the pyrolysis of the polymer *Bull Chem Soc Jap* 1963 36 905-909
- NORBERG K A Drug induced changes in catecholamine levels in the sympathetic adrenergic ganglion cell and terminals. A histochemical study *Acta physiol scand* 1963 65 221-231
- Transmitter histochemistry of the sympathetic adrenergic nervous system *Brain Res* 1967 (in press)
- NORBERG K A and B HAMBFRGER The sympathetic adrenergic neuron. Some characteristics revealed by histochemical studies on the intraneuronal distribution of the transmitter *Acta physiol scand* 1964 63 Suppl 239 1-42
- NORBERG K A P L RITZEN and U UNGERSTEDT Adrenergic innervation of the male reproductive ducts in some mammals. 1 The distribution of adrenergic nerves *Z Zellforsch* 1967 70 278-286
- NORBERG K A M RITZEN and U UNGERSTEDT Histochemical studies on a special catecholamine containing cell type in sympathetic ganglia *Acta physiol scand* 1966 67 260-270
- OLSON L Outgrowth of sympathetic adrenergic neurons in mice treated with a nerve growth factor (NGF) *Z Zellforsch* 1967 (in press)
- OWMAN C and N O SJÖSTRAND Short adrenergic neurons and catecholamine containing cells in vas deferens and accessory male genital glands of different mammals *Z Zellforsch* 1963 66 300-320
- PHARMACIA (Uppsala, Sweden) Vattenbestämning enligt Karl Fischer
- QUASTEL J H Molecular transport at cell membranes *Proc roy Soc B* 1963 163 189-196
- RICHARDS ON K C The fine structure of the albino rabbit iris with special reference to the identification of adrenergic and cholinergic nerves and nerve endings in its intrinsic muscles *Amer J Anat* 1964 114 173-203
- RITZEN M Quantitative fluorescence microspectrophotometry of catecholamine formaldehyde products *Exp Cell Res* 1966 44 505-510
- Cytochemical identification and quantitation of biogenic monoamines M.D Thesis Stockholm 1967
- ROSS S B and A L RENYI Blockin<sub>g</sub> action of sympathomimetic amines on the uptake of tritiated noradrenaline by mouse cerebral cortex tissues *in vitro* *Acta pharmacol* 1964 21 226-239
- Active uptake of tritiated metaraminol by mouse brain slices *in vitro* *Life Sci* 1966 5 639-647
- SACHS C Demonstration of metaraminol in nervous and non nervous tissues by a histochemical fluorescence reaction *Biochem Pharmacol* 1963 14 1773-1778
- SCHANBERG L S Passage of drugs across body membranes *Pharmacol Rev* 1962 14 501-530
- SCHWINK A and R WETZSTEIN Die Kapillaren im Subcommissuralorgan der Ratte. Elektronenmikroskopische Untersuchungen an Tieren verschiedenen Lebensalters *Z Zellforsch* 1966 70 56-88

- JONES O G Fluorescence method for the histochemical demonstration of monoamines. Fluorescence studies on biogenic monoamines and related compound condensed with formaldehyde *Histochemie* 1967a 8: 288—296
- Further studies on the specificity of the histochemical fluorescence method for the demonstration of catecholamines *Acta histochem* 1967b 6: 1—11
- Binding of catecholamines and related compounds to proteins after formaldehyde fixation *Histochemie* 1967c 8: 122—130
- JOHANSSON C and J. J. Microspectrofluorometric identification of metaraminol in catecholaminergic neurons *Acta physiol scand* 1966 67: 1—11
- KENNEDY D J and J. L. Reduction of the noradrenaline content of the stimulation *Acta physiol scand* 1963 17: 201—208
- KUROKAWA M and M. KATO Distribution of sodium plus magnesium ATPase and triphosphatase activity in isolated nerve endings *J* 1965 97: 831—844
- KUON F R and J. L. The rat brain: A stereotaxic atlas. Williams and Wilkins 1963
- LANDERS J W and L. CONZALEZ and W. PALUTKE Morphology of rat cerebral capillaries *Lab Invest* 1962 11: 123—133
- LICHTENSTEIN W and H. U. Uptake of exogenous catecholamines by monoamine oxidizing neurons of the central nervous system. Uptake of catecholamines by neurotoxic infundibular neurons *J Pharmacol exp Ther* 1964 187: 100—108
- LINDMAR R and E. M. Die Wirkung von Pharmaka auf die Elimination von Noradrenalin in der Perfusionssysteme und die Noradrenalinaufnahme in das isolierte Herz *Naunyn-Schmiedeberg's Arch exp Path Pharmacol* 1961 24: 119—122
- LUNDAHL P and R. S. Uptake of biogenic amines by two different mechanisms present in adrenergic granules *Brit J Pharmacol* 1964 (in press)
- MALMSTROM T Studies on adrenergic nerves. The use of rat and mouse for direct observations on their physiology and pharmacology at cellular and subcellular levels *Acta physiol scand* 1965 61 (Suppl 218): 1—93
- MALMSTROM J and C. S. Direct studies on the disappearance of the transmitter and the changes in the uptake storage mechanisms of degenerating adrenergic nerves *Acta physiol scand* 1965 61: 211—227
- MILLMAN H and R. ROBINSON *Practical neurochemistry* J. A. Churchill, London 1964
- MURPHY J and D. M. SMITH *Aquarium* Interscience, New York 1965

- MUROF K and K OKAWA The determination of the water content of the low polymers of formaldehyde by the Karl Fischer Method I The determination of the adsorbed water and bound water formed by the pyrolysis of the polymer *Bull Chem Soc Jap* 1963 36 965—969
- NORBERG K A Drug induced changes in catecholamine levels in the sympathetic adrenergic ganglion cell and terminals A histochemical study *Acta physiol scand* 1965 65 221—231
- Transmitter histochemistry of the sympathetic adrenergic nervous system *Brain Res* 1967 (in press)
- NORBERG K A and B HANBERGER The sympathetic adrenergic neuron Some characteristics revealed by histochemical studies on the intra neuronal distribution of the transmitter *Acta physiol scand* 1964 63 Suppl 238 1—12
- NORBERG K A P L RUSLEY and U UNGERSTEDT Adrenergic innervation of the male reproductive ducts in some mammals I The distribution of adrenergic nerves *Z Zellforsch* 1967 76 276—296
- NORBERG K A M RITZEN and U UNGERSTEDT Histochemical studies on a special catecholamine-containing cell type in sympathetic ganglia *Acta physiol scand* 1966 67 260—270
- OLSON L Outgrowth of sympathetic adrenergic neurons in mice treated with a nerve growth factor (NGF) *Z Zellforsch* 1967 (in press)
- OWMAN C and N O SJÖSTRAND Short adrenergic neurons and catecholamine containing cells in vas deferens and accessory male genital glands of different mammals *Z Zellforsch* 1965 66 300—320
- PHARMACIA (Uppsala Sweden) Vattenbestämning enligt Karl Fischer
- QUASTEL J H Molecular transport at cell membranes *Proc roy Soc B* 1965 163 169—196
- RICHARDSON K C The fine structure of the albino rabbit iris with special reference to the identification of adrenergic and cholinergic nerves and nerve endings in its intrinsic muscles *Am J Anat* 1964 114 173—205
- RITZEN M Quantitative fluorescence microspectrophotometry of catecholamine formaldehyde products *Exp Cell Res* 1966 44 50—520
- *Cytochemical identification and quantitation of biogenic monoamines* M.D Thesis Stockholm 1967
- ROSS S B and A L RYAN Blocking action of sympathomimetic amines on the uptake of tritiated noradrenaline by mouse cerebral cortex tissues *in vitro* *Acta pharmacol* 1964 21 226—239
- Active uptake of tritiated metaraminol by mouse brain slices *in vitro* *Life Sci* 1966 5 639—647
- SACHS C Demonstration of metaraminol in nervous and non nervous tissues by a histochemical fluorescence reaction *Biochem Pharmacol* 1965 14 1773—1778
- SCHANKER L S Passage of drugs across body membranes *Pharmacol Rev* 1962 14 501—530
- SCHWINK A and R WETZSTEIN Die Kapillaren im Subcommisuralorgan der Ratte Elektronenmikroskopische Untersuchungen an Tieren verschiedenen Lebensalters *Z Zellforsch* 1966 73 86—88



- SFEDVALL C Short term effects of reserpine on noradrenaline levels in skeletal muscle *Acta physiol scand* 1961 **12** 101—108
- STITZEL R and P LINDHORE Effect of reserpine and monoamine oxidase inhibition on the uptake and subcellular distribution of  $^3H$  noradrenaline *Brit J Pharmacol* 1967 **29** 99—104
- STJERNBERG L Studies of catecholamine uptake, storage and release mechanisms *Acta physiol scand* 1961 **12** Suppl 228 1—97
- STROMBLAD B C R and M NICKERSON Accumulation of epinephrine and norepinephrine by some rat tissues *J Pharmacol exp Ther* 1961 **134** 131—139
- TAYLOR J Contribution à l'étude des connexions des neurones moteurs du système nerveux autonome *Ann de Sciences Naturelles Zoologie* 1963 **7** 113—674
- THIRME G Small tissue dryers with high capacity for rapid freeze drying *J Histochem Cytochem* 1963 **13** 380—389
- TITUS I and H J DICKLER The mechanism of uptake of norepinephrine *Pharmacol Rev* 1966 **18** 325—335
- TITUS I, N MATTHEWS H I SIEGEL and B B BRODIE The effects of isomethylinpramine on uptake of  $^3H$  norepinephrine in the heart *J Pharmacol exp Ther* 1966 **152** 469—477
- UNRHEIT W W R H BURRIS and J L STALLER *Manometric techniques and tissue metabolism* Burgess Publ Minneapolis 1951
- WALKER J F *Formaldehyde* Reinhold Publ London 1961
- WELL-MALHERBE H C WHITBY and J AXELSON The uptake of circulating  $^3H$  norepinephrine by the pituitary gland and various areas of the brain *J Neurochem* 1961 **6** 3—64
- WHITTAM R The interdependence of metabolism and active transport. In *The cellular functions of membrane transport* (ed J F HORRIGAN) Intext Hall Englewood Cliffs 1964 149—154
- WHITTAM R and D M BLOOM Respiratory control by an adenosine triphosphatase involved in active transport in brain cortex *Biochem J* 1964 **92** 117—129
- WILBRANDT W and T ROSENBERG The concept of carrier transport and its correlates in pharmacology *Pharmacol Rev* 1961 **13** 109—183
- WIRGIN C Studies in lipid mobilization with special reference to morphological and histochemical aspects *Acta physiol scand* 1963 **12** Suppl 232 1—16
- WIRGIN C and B HANBERGER A note on catecholamines in brown fat *Nature* 1967 (in press)
- WOLFE J Beiträge zur Ultrastruktur der Kapillaren in der Nierne *Celluläre Z Zellforsch* 1963 **10** 109—131
- YOSHIDA H, J NAKATA, K KASHIKI and R ISHIZUMI Studies on active transport of  $^3H$  dehydrotetrahydroxyphenylamine into brain slices *J Pharmacol* 1963 **15** 1—9

## FIGURES

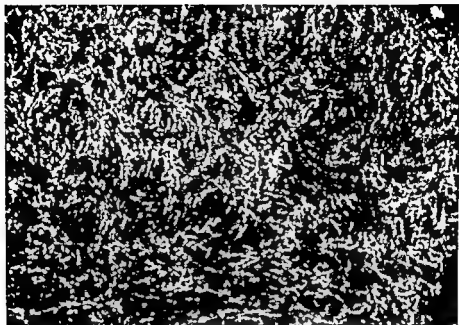


Fig. 1 Slice of vas deferens untreated rat incubated in standard medium without drugs for 1 h. Nerve terminals with strong fluorescence variations.  $\times 100$

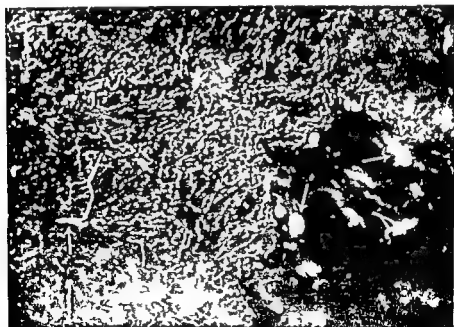


Fig. 2 Slice of vas deferens from a rat treated with 100  $\mu$ g/ml Nal. Nerve terminals as well as a bundle of nonterminal axons ( $\rightarrow$ ) exhibiting strong autofluorescence.  $\times 100$



Fig. 3 Slice of vas deferens untreated rat incubated in standard medium without drugs for 2 h. Above the strongly fluorescent nerve terminals a bundle ( $\rightarrow$ ) of non-terminal axons with weak fluorescence  $\times 160$



Fig. 4 Slice of vas deferens reserpine pretreated rat incubated with  $\alpha$  methyl N1 (0.1  $\mu$ g/ml). Nerve terminals with fluorescent varicosities and on the right both a single and bundles of non-terminal axons  $\times 210$



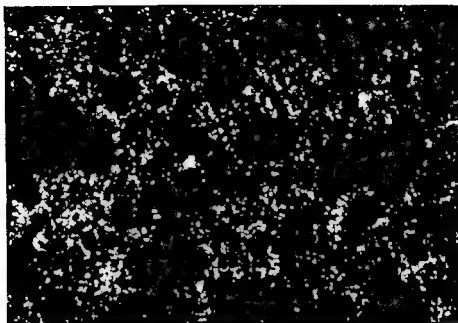


Fig 6 Slice of nucleus caudatus putamen reserpine-pretreated rat incubated with  $\alpha$ -methyl DA ( $0.1 \mu\text{g/ml}$ ) High density of strongly fluorescent varicosities of DA nerve terminals The dark areas are non fluorescent nerve cell bodies  $\times 400$



Fig 7 Slice of nucleus caudatus putamen reserpine-pretreated rat incubated with  $\alpha$ -methyl NA ( $1 \mu\text{g/ml}$ ) On the left DA nerve terminals which have accumulated the amine to a varying degree so that the peripheral part of the slice (A) has higher fluorescent intensity Single fluorescent non-terminal axons in the tissue



Fig 8 Slice of nucleus caudatus putamen reserpine pretreated rat incubated with methyl NA (1  $\mu\text{g}/\text{ml}$ ) Extremely dense network of nerve terminals with distinct varicosities. The large dark areas are trunks of non terminal axons and the small ones are nerve cell bodies  $\times 900$



Fig 9 Slice of cerebral cortex untreated rat incubated with methyl NA (1  $\mu\text{g}/\text{ml}$ ) Several strongly fluorescent nerve terminals with distinct varicosities  $\times 230$



Fig 10 Slice of hypothalamic area untreated rat incubated in the standard medium without drugs for 30 min. A network of fine to fairly thick terminals of various densities. Arrows point to specific features.



Fig 11 Slice of hypothalamic area reserpine pretreated rat incubated with methyl NA (1  $\mu\text{g}/\text{ml}$ ) Several nerve cell bodies with strong fluorescence  $\times 410$



Fig. 18 Slice of hypothalamic area untreated rat incubated with  $\alpha$ -methyl NA ( $3 \mu\text{g/ml}$ ) Fluorescent cells in the endothelial wall are confined to the capillaries whereas the larger vessel has no such cells  $\times 10$



Fig. 19 Slice of nucleus caudatus putamen reserpine and malachite green pretreated rat incubated with DA ( $1 \mu\text{g/ml}$ ) Fluorescent cells in the endothelial wall At (→) a typical pericyte bulging out  $\times 370$



Fig. 20 Slice of hypothalamic area reserpine-pretreated rat incubated with  $\alpha$ -methyl NA ( $1 \mu\text{g/ml}$ ) Specific fluorescence in the endothelial wall (→) a typical pericyte  $\times 370$



Fig. 21 Slice of cerebral cortex untreated rat incubated with  $\alpha$ -methyl NA ( $10 \mu\text{g/ml}$ ) After freeze-drying and formaldehyde gas treatment, embedded in Araldite<sup>®</sup> as 1 section at  $1 \mu$  (These specimens were kindly prepared by Dr







ACTA PHYSIOLOGICA SCANDINAVICA

*Supplementum 296*

THE RELATION BETWEEN THE  
ELECTRICAL AND MECHANICAL  
ACTIVITY OF HUMAN INTERCOSTAL  
MUSCLES DURING VOLUNTARY  
INSPIRATION

HELSINKI 1967



ACTA PHYSIOLOGICA SCANDINAVICA

*Supplementum 296*

FROM THE INSTITUTE OF PHYSIOLOGY  
UNIVERSITY OF HELSINKI  
HELSINKI FINLAND

THE RELATION BETWEEN THE  
ELECTRICAL AND MECHANICAL  
ACTIVITY OF HUMAN INTERCOSTAL  
MUSCLES DURING VOLUNTARY  
INSPIRATION

BY  
ANTTI A VILJANEN

HELSINKI 1967



## PREFACE

This investigation was carried out at the Institute of Physiology University of Helsinki. The problem was suggested by the Head of the Institute, Professor P. M. Bergström M.D. who generously placed the equipment of the institute at my disposal. I wish to express my deep gratitude for his constant advice and encouragement as well as for the critical comments he made during the course of the work.

I am also greatly indebted to Docent Hakan Poppius M.D. for his valuable advice in regard to both the theoretical and technical aspects of the study.

The support given by Associate Professor H. S. S. Särjäs Ph.D. M.D. was very welcome and encouraging during the whole investigation. The valuable remarks of Docent Eira Hirsjärvi M.D. on some special topics and the useful advice of Mr. Simo Oja Ph.D. M.D. have been greatly appreciated.

My sincere thanks are due to all members of the staff of the Institute of Physiology and especially to Mr. Roger W. T. Blafield C.E. for his skillful technical assistance and Mr. Pekka Halttunen B.M. for his continuous interest and valuable assistance during time consuming experiments. I am also grateful to Mr. Martti Halumäki M.D., Mr. Kyro Niemi M.D., Mr. Seppo Pyrhonen B.M. and Mr. Lauri Lütinen.

The invaluable help provided by Mr. Erika Kurenniemi who developed the procedures for the mathematical computations has placed me greatly in his debt. Grateful acknowledgement is given to Mr. Timo Mäkeläinen M.Phil.Sc., of the Institute of Statistics University of Helsinki for advice in preparation of all the statistical analyses.

My thanks are also due to Mr. Yrjö Salorinne B.M. for assistance in formulation of the English text and to Mr. Fred Fewster for checking the manuscript.

I wish to extend my thanks to Mrs. Marjatta Kuru for typing the manuscript.

My sincere gratitude is expressed to the courageous experimental subjects young medical students, and the many other persons who offered their help during the course of the investigation

This study has been supported by grants from the Finnish Anti Tuberculosis Association and the Central Board of Research Councils in Finland

My wife is entitled to my warmest gratitude, her untiring support and understanding made the successful conclusion of the work possible

Helsinki April 1967

ANTTI A. VILJANEN

## CONTENTS

INTRODUCTION	7
SURVEY OF LITERATURE	8
MATERIAL AND METHODS	13
Material	13
Recording of electrical activity	13
Mechanical activity of inspiratory muscles	16
Measurement of tidal volume	16
Measurements of alveolar relaxation pressure and oesophageal pressure	16
Measurement of the movements of the thoracic cage	17
Calculation of the mechanical activity of the inspiratory muscles	17
CO <sub>2</sub> stimulation	19
Experimental conditions	20
Statistical methods	21
RESULTS	23
Experiments under normal conditions	23
Electromyogram impulse frequency at the end of inspiration and inspiratory muscular work	23
Total electromyogram impulse number and the first time integral of the inspiratory muscular work	26
Electromyogram impulse frequency four seconds after the end of inspiration and the alveolar relaxation pressure	27
Electromyogram impulse number and the pressure impulse produced by inspiratory muscles	29
Experiments during CO <sub>2</sub> stimulation	30
Comparison of the electromyograms recorded with surface electrodes and needle electrodes	III
Movements of the thoracic cage during inspiration	32
DISCUSSION	36
SUMMARY	43
REFERENCES	45
APPENDIX	51





## INTRODUCTION

In studies of the regulation of human respiration one general opinion often expressed is that the movements involved express the intrinsic activity of the respiratory centres. However knowledge is necessary of the relations existent between the neuronal activity and the mechanical activity of the respiratory muscles. It has earlier been demonstrated that in animals the electrical activity of the nervus phrenicus bears a linear relation to the activity of the diaphragm recorded electromyographically (DITTLER 1910, DITTLER & GAPTEN 1913, GASSER & NEWCOMER 1921). If knowledge is to be gained of the activity of human respiratory centres it is first necessary to know the laws which bind the electrical activity of the respiratory muscles and the corresponding mechanical activity.

In this study an attempt was made to solve the problem posed by the relations between the electrical and mechanical activity of human intercostal muscles during voluntary inspiration. Experiments were also conducted with CO<sub>2</sub> stimulation. The electrical activity was registered during inspiration by means of a sample electromyogram of the external intercostal muscles. Since the mechanical activity of the external intercostal muscles cannot be measured directly in man the total mechanical activity of the inspiratory muscles was calculated instead.

It may be that knowledge of the relationships which exist between the electrical and mechanical activities determined in this way will lead to further progress in studies which aim at solution of the problems connected with the neuronal regulation of respiration. Moreover the information so gained may be applicable in the monitoring of respiratory functions.

## SURVEY OF LITERATURE

It has been shown that an integrated electromyogram registered with surface electrodes bears a linear relationship to the tension produced by a voluntary isometric contraction in human muscle (BAYER & FLECHTENGLACHER 1950 INMAN et al 1952 LITFOLD 1952). Furthermore it has been reported that integrated action potentials recorded by surface electrodes from the human gastrocnemius muscle during voluntary contractions are directly proportional to the tension when the velocity of shortening or of lengthening remains constant. At a constant tension the electrical activity increases linearly with the velocity of shortening (BIGLAND & LITFOLD 1954a). A linear relationship has also been demonstrated between the discharge frequency of the electromyogram recorded from the forefinger abductor muscle applying a non-selective electrode technique and the isometric tension produced by abduction (JALAVISTO et al 1953). JALAVISTO et al have also shown that the impulse frequency increases with velocity during isotonic shortening.

BERGSTROM in his studies of the human forefinger abductor muscle by a non-selective surface electrode method has later shown that 1) a linear relationship exists between the number of motor impulses and the integrated electromyogram activity (1959a) 2) the number of motor impulses in nearly isometric contractions (leichte Aktionen) bears a linear relationship to the mechanical force impulse (1958) 3) the discharge frequency of the electromyogram is directly proportional to the kinetic energy produced by the abduction with the kinetic energy calculated from a movement of a pendulum after a touch of the forefinger and the electromyogram frequency recorded during 0.01 second immediately prior to the moment of detachment (1959b) 4) the impulse frequency of the electromyogram is directly proportional to the degree of muscle shortening at different loadings of the contractions and the amount of work produced by unloaded contraction and furthermore to the estimated work the muscle must perform for attainment of the

abduction degree (1959 c) and 5) the number of electromyogram impulses is directly proportional to the physical action (energy  $\propto$  time dimension  $g \times cm \propto s^{-1}$ ) of the mechanical effect of the motion (1959 b) Subsequently in electromyogram recordings from the human musculus soleus with two copper wire electrodes it has been shown that 1) the integrated electrical activity bears a linear relationship to the number of electromyogram impulses recorded by an electrical counter, 2) in isometric contraction this tension is directly related to the action potential count or frequency 3) in isotonic contractions there is an almost linear relationship between total action potential count and work 4) and if this distance and the duration of contraction are constant, the action potential count is proportional to the average tension (CLOSE NICKEL & TODD 1960)

In evaluation of the results cited it is important to note that even if a linear relationship is found between the tension and the electrical activity of human skeletal muscle by the application of a non selective technique when in fact the activity of several motor units is being recorded the situation changes when the technique is sufficiently selective to give the activity of a single motor unit (JALAVISTO et al 1938 BIGLAND & LIPPOLD 1954 b) BIGLAND & LIPPOLD have further indicated that the frequency of one unit is related to the strength of contraction giving an S shaped curve

A number of research workers (ADRIAN & BRONK 1929 SMITH 1934 LINDSLEY 1935 GILSON & MILLS 1941 BIGLAND & LIPPOLD 1954 b) have reported on the absence of any electrical activity in the relaxed muscle

The gradation of the voluntary contraction of skeletal muscle in man has been shown to originate in changes in the discharge frequency, the duration of motor discharge and the number of active motoneurons (ADRIAN & BRONK 1929 SMITH 1934 LINDSLEY 1935, GILSON & MILLS 1941 TOKIZANE KAWATA & TOKIZANE 1952 BIGLAND & LIPPOLD 1954 b) This has further been demonstrated in gradation of the contraction of the striated skeletal muscle in animals (ADRIAN & BRONK 1929 BRONK & FERGUSON 1935 ATKINSON, BROWN & GESELL 1940 GESELL & ATKINSON 1943)

The importance of the external and internal intercostal muscles to respiration has also posed a problem According to Hamberger's theory the external intercostal muscles and the intercartilaginous portion of the internal muscles are inspiratory and the interosseus portion of the internal muscles expiratory (cf CAMPBELL 1958)

Electrical recordings from the intercostal nerves (BRONK & FERGUSON 1935) and muscles (ANDERSON & LINDSLEY 1935) in cats showed the

external muscles to be inspiratory, and internal intercostal muscles expiratory. A similar finding was made by CHENNELS (1957) in experiment on both cats and rats. GESELL (1936) has shown that there are numerous individual variations in dogs. CAMPBELL (1955a) has studied young men electromyographically with both surface and needle electrodes and has reported that in most male subjects the intercostal muscles of the lower intercostal spaces (5-9) contract during quiet inspirations. GREEN & HOWELL (1959) arrived at the same conclusion. TAYLOR (1960) on the basis of electromyogram studies with fine needle electrodes found the human external intercostal muscles to be purely inspiratory in action whereas the internal layer is expiratory except in regard to the parasternal part which is inspiratory. According to him in quiet inspiration only the parasternal region of the internal intercostal muscle is active but the external intercostal in the supine position is not. KOPFKE et al. (1955) who employed needle electrodes and an oscilloscope have stated that only in the first intercostal muscle is there electrical activity in quiet breathing they were unable to separate the various layers of the intercostal muscles. JONES, BRADSHAW & PAULY (1953), and JONES & PAULY (1957), who used surface electrodes for examination of muscles in the parasternal and mid clavicular regions discovered no rhythmic electrical activity in the intercostal muscles during quiet breathing. JONIZAN et al. (1952) who used needle electrodes reported an absence of rhythmic electrical activity in the four upper intercostal muscles during quiet breathing. With a deepening of inspiration they detected a clear increase in the electrical activity of the intercostal muscles (JONES et al. 1953 CAMPBELL 1955a JONES & PAULY 1957 DRAIFER LADEROOFD & WHITTERIDGE 1957 KOPFKE et al. 1958 TAYLOR 1960).

With respect to animals it has been possible to demonstrate that the gradation of the contraction of intercostal muscles is dependent upon the recruitment of motoneurons, their action frequency and the action time (BRONK & IERELSON 1933 CRESILL ATRINSON & BROWN 1941, CRESILL HUNTER & JILLIE 1949). The same mechanism is involved in regulation of the contraction of the diaphragm (GIESILL et al. 1941 IITTS 1942). Nevertheless some research workers have stated that the synchronization of motor impulses is important to regulation of the contraction in the muscles of the diaphragm (DITTLER 1910 WINTERSTEIN 1911 DITTLER & GARTEN 1912 GASSER & NEWCOMER 1921, GASSER 1925 ADRIAN & BRONK 1928 HESS & WYSS 1936 Bishop & BLOCHINGER 1936 PULANT 1937) although many of them are of opinion that impulse synchronization occurs only with increasing

dyapnoe (DITTLER 1910, GASSER 1929, ADRIAN & BROOK 1924, WISS 1939) WACHOLDER & MCKINLEY (1929) have stated that no synchronization exists in regulation of the contraction of the diaphragm

Although extensive studies have been made of the electrical activity of the respiratory muscles in animals relatively few research workers have compared the mechanical activity with the electrical activity measured either by integration of the action potentials or by counting the impulses from electromyograms FINE, NGAI & DUNCAN (1958) have shown in the decerebrate cat that the integrated electromyogram activity of the diaphragm increases approximately linearly with the inspiratory load RAMOS & ARJOVA (1956) with Nembutal anaesthetized rabbits found that the breathing activity measured by mechanical or electrical integration of the electromyograms of the diaphragm bears a linear relationship to oxygen consumption They determined the oxygen consumption with a respirometer of Collins type the electromyogram of the diaphragm was registered with small steel serrated electrodes fixed through the abdominal cavity on to the lower surface of the diaphragm WOLDRING (1965) using anaesthetized cats with open thorax studied the activity of the diaphragm and abdominal muscles as a function of lung volume and arterial CO<sub>2</sub> tension intramuscular electrodes and electronic integration of action potentials were applied He demonstrated that the integrated electrical activity of the diaphragm bears a linear relationship to the arterial CO<sub>2</sub> tension when the lung volume is the independent variable and moreover that the relationship between the diaphragm activity and the lung volume at a given arterial CO<sub>2</sub> tension is given by a straight line with a negative slope GILL (1963) examined the effect of the end tidal CO<sub>2</sub> on the discharge of phrenic motoneurons in decerebrate cats, and found that both the recruitment of additional units and the increase in discharge of phrenic units already active play a role in augmenting the phrenic discharge as the end tidal CO<sub>2</sub> is increasing BERGSTROM & KERTTOLA (1961) with narcotized rats have shown that the inspiratory work determined by recording the changes in the inspired air volume and in the intrapleural pressure of the system in the course of inspiration bears a linear relationship to the impulse frequency of the electromyograms measured at the end of the inspiration They also demonstrated that the total number of action potentials is in linear relationship to the first time integral of the inspiratory work The electromyogram was registered with platinum thread brush electrodes and needle electrodes from the external intercostal muscles with the aid of a cathode ray oscillograph and amplifier

In many human experiments electromyograms from the respiratory

muscles have been registered simultaneously with some mechanical functions of respiration, although comparatively few studies have been concerned with numerical evaluation of the relationships existent between the electrical and mechanical activity of the respiratory muscles. ROS SIFF et al (1956) were probably the first to register an electromyogram from the diaphragm: they made use of needle electrodes and recorded rhythmic electrical activity which increased during inspiration and almost totally vanished during expiration. AGOSTONI, SANT'AMBROGIO & DEL PORTILLO GARRASCO (1960) employed an oesophageal bipolar electrode for study of the electrical activity of the vertebral part of the diaphragm in man, and registered simultaneously the spirogram and/or the intrathoracic and gastric pressure. They found that during inspiration the electrical activity of the diaphragm was approximately related to the transdiaphragmatic pressure as the activity found during graded inspiratory efforts at relaxation volumes. By application of almost the same methods as AGOSTONI et al (1960) PETIT, MILIC I MILIC & DELHEZ (1960) also found synchronization of the diaphragmatic activity with the respiratory variations of trans-diaphragmatic pressure.

DELHEZ, PETIT & MILIC I MILIC (1959) (cf PETIT, DELHEZ & TROQUET 1967) have shown that the integrated electromyogram activity of the recti abdomini in man is directly proportional to the calculated tension of abdomen muscles. DELHEZ et al (1965) and DELHEZ, PETIT & BOTTIN (1965) have reported results from human experiments that the integrated electromyogram activity of the diaphragm bears a linear relationship to the calculated inspiratory work. The electromyogram from the diaphragm was recorded with oesophagus electrodes during individual inspirations and the total inspiratory work was calculated from dynamic pulmonary elastic and thoracic elastic works.

CAMPBELL & GRIFFIN have demonstrated that electromyogram activity registered with surface electrodes from the external oblique (1957 a, b) and rectus abdominis muscles (1953 a) is directly proportional to the intra-abdominal pressure during voluntary graded expiration when the chest volume remains constant. GROVBY & SKOUDY (1960) with healthy males and patients with chronic asthma and emphysema have examined the electromyogram activity of the diaphragm with oesophagus electrodes: they found that during quiet breathing the inspiratory diaphragmatic discharge was generally greater in patients than it was in healthy persons and further that the diaphragmatic activity definitely increased in the healthy persons when forced breathing was carried out.

## MATERIAL AND METHODS

### Material

The experimental subjects were 14 young, healthy males aged from 20 to 24 years. As electromyogram recordings were made by means of surface electrodes persons with thick subcutis were excluded. The electrical activity of the intercostal muscles and the mechanical activity of the inspiratory muscles were registered simultaneously during voluntary inspirations. Analysis was made of 1055 voluntary inspirations of which 141 were registered during  $5 \pm 0.4\%$  CO<sub>2</sub> stimulation and 155 after it. In addition 1033 voluntary inspirations were registered for determination of the alveolar relaxation pressure as a function of volume.

### Recording of electrical activity

The electrical activity of the intercostal muscles was registered electromyographically. The recordings were made in a Faraday cage in a quiet room. The electromyograms were generally registered with bipolar surface electrodes. The electrode distance was 25 mm and the diameter of an electrode 7 mm. The ordinary electrodes were pieces of felt clamped on plastic and fixed with Tensoplast to the skin which had been cleaned with ether. Before the experiments the felt was moistened with electrode fluid (Mungograph electrode liquid Elema — Schönander). The choice of this type of electrode is the most suitable followed examination of a large number of surface electrodes. For determination of the effect of the summation and subtraction of the action potentials in this electrode on the number and discharge frequency of electromyogram impulses, simultaneous registrations with a DISA concentric needle electrode (diameter 0.35 mm) were made from the same intercostal muscle. The two electromyograms were mutually compared. From elec



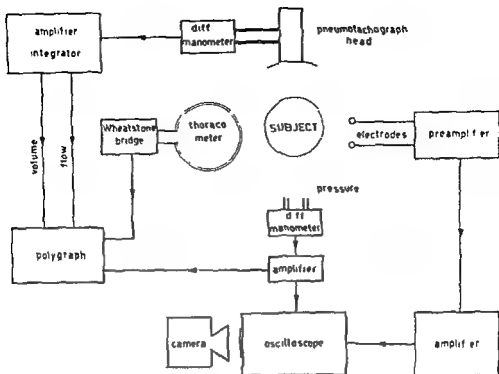


Fig 1 a

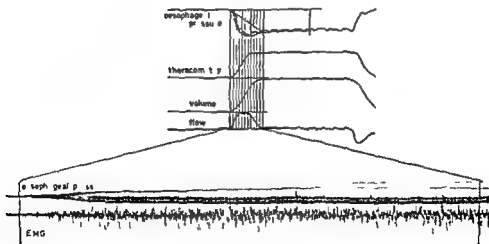


Fig 1 b

Fig 1 a) recording system

b) example of the various simultaneous registrations during experiments (tidal volume 2.9 l duration of inspiration 2.70 sec oesophageal pressure change measured at the end of inspiration 13.1 cm H<sub>2</sub>O)

trodes the electromyogram impulses were led to a Tonnes differential preamplifier on to a Tetronix 502 oscilloscope and were filmed from the screen with a Physiologic Milano (Arduine) camera. The gain of the preamplifier was 100, and the sensitivity of the oscilloscope generally 1 cm/100 mV. The speed of the film was 152 mm/sec. For timing of the electromyogram with the recordings of respiratory movements the oesophagus pressure was led simultaneously to the other oscilloscope channel from which the beginning of each inspiration could be seen. In view of the impossibility of determining the total number of action potentials in the action currents of all the motoneurons of the intercostal muscles recruited during inspiration a sample was taken from the electrical activity of muscles: this was usually taken from the sixth intercostal muscle near the right mid clavicular line as during the preliminary investigations this region had proved to be the most satisfactory for recordings (VILJANEN et al 1965). The subjects were in a supine position during examination. The size and position of the electrodes were kept constant, as were the amplification of the action potentials and other experimental conditions. It can be assumed that a sample of the electrical activity of the intercostal muscles obtained in this way represents the total electrical activity (BERGSTROM & KERTTULA 1961). The following determinations were made by visual counting from the film: 1) the total number of electromyogram impulses (n number) during inspiration; 2) the electromyogram impulse frequency during a 1/3 second interval at the end of inspiration, in this study entitled the impulse frequency of dynamic phase ( $f_d$ ) and 3) the electromyogram impulse frequency determined four seconds after the end of inspiration, the subject holding his breath with the glottis open, in this study entitled the impulse frequency of the static phase ( $f_s$ ). 4) the increase in the number of electromyogram impulses during one and the same inspiration calculated as a function of time for comparison with the mechanical activity of respiratory muscles also measured as a function of time.

The electromyogram impulses were counted by two persons and a third checked the counting by arbitrarily selected samples. The differences between the results counted by two persons were  $\pm 1\%$ . From the electromyogram there were counted all the impulses which were distinguishable from the baseline irrespective of their location and amplitude. In principle the method of counting was the same as that used by BERGSTROM (1959 a).

The mechanical activity of inspiratory muscles was determined from the alveolar relaxation pressure ( $P_{al}$ ), the intra oesophageal pressure ( $P$ ) and the inspiratory tidal volume ( $V$ ). The movements of the thoracic cage were also registered.

## Measurement of tidal volume

The recording of the inspiratory volume ( $V$ ) and flow ( $\dot{V}$ ) was effected with a Fleischpneumotachograph head (cf ILFISCH 1925 1956) No 204 (in which 5 mm H<sub>2</sub>O equals 1.00 l/sec and 10 mm H<sub>2</sub>O equals 3.19 l/sec) an Elema Schonander differential manometer EMT 572 No B 111 (range 0-50 mm H<sub>2</sub>O), an electrical integrator Type EM1 573 No B 111 and a four channel polygraph Mingograph 42. In each experiment the volume was calibrated with a piston pump of known volume.

## Measurements of alveolar relaxation and oesophageal pressure

The alveolar relaxation and intra oesophageal pressure were registered with a Sanborn's differential pressure transducer model 270, the differential pressure range being  $\pm 400$  mm H<sub>2</sub>O.

Determination of the alveolar relaxation pressure was made by getting the subject under normal conditions to inspire a given volume and then to relax as completely as possible against an obstructed airway (ROHMER 1916 PARRY et al 1946). The volume and pressure were led to the polygraph and measured manually from the paper. For each subject an average of  $74 \pm 19$  pressure-volume points was registered and the alveolar relaxation pressure ( $P_{al}$ ) was determined from the  $\pi$  as a function of the volume ( $V$ ).

The intra oesophageal pressure ( $P$ ) was measured with an air containing latex balloon connected through the nose by a catheter to the manometer (BURTENDIJK 1949 FRY et al 1952 MEAD et al 1955 CPANE HAMILTON & ARFELDT 1956 PETT & MILIC EMILI 1958 SCHILDER HIATT & FRY 1959). The balloon was 10 cm in length the perimeter was 24 mm and the thickness of the wall of the balloon was approximately 0.15 mm the length of the polyethylene catheter 60 cm and the internal diameter 2.5 mm. In the experiments there was 0.25-1.0 ml of gas in the balloon as it has proved that the error of measurement is small within this range (PETT & MILIC I MILI 1958). During measurement the

balloon was in the lower third of the oesophagus (MILIC EMILI MEAD & TURNER 1964)

A water manometer was employed for pressure calibration during each experiment

### *Measurement of movements of the thoracic cage*

Movements of the thoracic cage were measured with a rubber tubing of 2.0 mm bore and 3.5 mm external diameter wrapped around the chest. The tube was filled with saturated  $\text{CuSO}_4$  liquid. The lengthening and narrowing of the column of  $\text{CuSO}_4$  liquid increased its electrical resistance which formed one arm of a balanced Wheatstone bridge, when the resistance varied it changed the balance of the bridge. The changes were amplified and recorded with the polygraph. The method is in principle the same as that applied by WADE (1954). The linearity of this transducer was good within the 0–10 cm range (in 50 measurements the linear correlation coefficient ( $r$ ) was 0.998).

### *Calculation of the mechanical activity of the inspiratory muscles*

The inspiratory muscles work against the following forces expressed in terms of pressure (ROHNER 1915, 1916; MEAD 1961; for symbols of MILIC EMILI MEAD 1964): 1) the elastic forces of the lung and chest

$$P_{el} = P_{(l)} + P_{(w)}$$

in which  $P_{(l)}$  is the elastic force of the lung and  $P_{(w)}$  is the elastic force of the chest; 2) the flow resistive forces ( $P_{res(f)}$ ) which are dependent on the resistance of gas flow ( $P_{res(g)}$ ) and on the non elastic resistance of the tissue deformation ( $P_{res(t)} + P_{res(w)}$ )

$$P_{res} = P_{(l)} + P_{(g)} + P_{res(t)}$$

where  $P_{res(t)}$  is the resistance of the non elastic deformation of the lung and  $P_{(w)}$  that of the chest; 3) the inertial forces ( $P_{in}$ ) which are dependent on the mass of the tissues and gases

$$P_{in} = P_{(l)} + P_{in(g)} + P_{(w)}$$

where  $P_{(l)}$  is the inertial force of the lung,  $P_{in(g)}$  that of the gas and  $P_{(w)}$  that of the chest.

The total force of the inspiratory muscles ( $P_{mus}$ ) is the equivalent of

the sum of the elastic forces flow resistive forces and inertial forces  
The equation of the total force of the inspiratory muscles is

$$P_m = P_{el} + P_{fr} + P_{in}$$

A large number of studies of normal lungs have indicated that the elastance is independent of breathing frequency (MEAD & WHITTENBERGER 1953 MEAD et al 1955, CHIRNIAK 1956 OTIS et al 1956, RAY et al 1957 BUTLER & SMITH 1957 DEFARES & DONLFRY 1960) This means that the alveolar relaxation pressure can be assumed to be equal to the total inspiratory elastic forces ( $P_{al}$ )

The flow resistive and inertial forces were determined by measurement of the intra oesophageal pressure as in many studies the changes in the intra oesophageal pressure correspond rather well to changes in the intra pleural pressure at least in the upright posture of the human being (DORNHORST & LUTHART 1952 LRY et al 1952 McILROY, MARSHALL & CHRISTIE 1954 ATTINGER MONROE & SIGAL 1956 BUTLER & SMITH 1957 FERRIS MEAD & FRANK 1959 HOWLES HONG & RAY 1959 MEAD & GAENSLE 1959 EHRLER 1960) In subjects studied supine the oesophageal pressure became less sub atmospheric than the intra pleural pressure it is concluded that this is attributable to oesophageal compression of the mediastinal structures in the supine posture (MEAD & GAENSLE 1959 HOWLES et al 1959 FERRIS et al 1959) In the present study the changes in oesophageal pressure were measured in supine subjects Nevertheless it is probable that the error in measurement is small since only the flow resistive and inertial forces of the lung were measured

For each inspiration the flow resistive and inertial forces of the lung were measured manually from the polygraph paper The increase in pressure was calculated as a function of time as follows the beginning and end points of the pressure curve were connected and this distance between this straight line and the actual pressure curve was measured at 0.25 sec intervals In principle this is the same method as that applied by NFERGAARD & WITZ (1927) for their measurements of the intra pleural pressure The calculated flow resistive and inertial forces of the lung in each inspiration were added to the respective alveolar relaxation pressure and the sum of the two pressures as a function of the time was written as equal to the forces against which the inspiratory muscles work during inspiration In pursuance of this procedure the flow resistive and inertial work effected against the tissues of the thorax and abdomen is not included However this work is comparatively little when very forced inspirations are avoided (OTIS FENN & RAY 1950 CHRISTIE 1953 AGOSTONI 1961)

If there is written

$$P_{dy} = P_{at(1)} + P_{in(1)} + P_{at(g)} + P_{i(g)}$$

the total force of inspiration muscles ( $P_{mu}$ ) can be expressed as the following equation

$$P_{mu} = P_{at(1)} + P_{dy}$$

In each inspiration pressure  $P_{mu}$  was determined as a function of time ( $P_{mu} = f(t)$ ) for the mathematical manipulation

When the force of inspiratory muscles ( $P_{mu}$ ) and the change of volume in the lung during inspiration are known as functions of time there can be calculated 1) the inspiratory muscular work ( $W$ ) during breathing which is

$$W = \int P_{mu} \times dV,$$

2) the first time integral of the inspiratory muscular work ( $H_i$ ) by application of the equation

$$H_i = \int W \times dt,$$

and 3) the pressure impulse of the inspiratory muscles ( $I$ ) by application of the equation

$$I = \int P_{mu} \times dt$$

all as functions of time. These can be related to the respective electrical activity of the intercostal muscles also expressed as a function of time.

In each inspiration the changes in pressure and volume were measured from the polygraph paper at intervals of 0.25 seconds. A computer ( $\mu$  Line) was employed in calculation of the equations. Thus the following parameters were obtained: 1) tidal volume ( $V$ ); 2) the force of the inspiratory muscles ( $P_{mu}$ ); 3) muscular work ( $W$ ); 4) the first time integral ( $H$ ) of the muscular work; and 5) pressure impulse ( $I$ ) all in digital form at 0.25 seconds intervals as a function of time.

### CO<sub>2</sub> stimulation

In the CO<sub>2</sub> stimulation experiments the six subjects breathed in through a flap valve from a plastic bag containing 2000 litres  $5 \pm 0.4\%$  CO<sub>2</sub> air and expired through another valve into the atmospheric air. The flow resistance of the valve was 1 litre/sec corresponding to 15 mm

H<sub>2</sub>O and 1.5 litres/sec corresponding to 31 mm H<sub>2</sub>O. The EMG from the external intercostal muscles, and the corresponding mechanical magnitudes of the normal state were recorded before CO<sub>2</sub> stimulation was started. When stimulation had continued for  $25 \pm 9$  min, similar registrations were made again. The recording period was  $12 \pm 2$  min. Immediately after this CO<sub>2</sub> stimulation was discontinued by switching the valve to normal atmospheric air. These registrations were repeated after  $14 \pm 2$  min. During the whole course of the experiment the position of the electrode, the necessary amplifications and other experimental conditions were kept constant. The subjects in this experiment were also in a supine position.

### Experimental conditions

The experiments were carried out in a quiet room and no recordings were made before the subjects had rested for at least half an hour. The experiments for measurement of the alveolar relaxation pressure lasted 1–1½ hours and those in which the electrical and mechanical activities during inspiration were measured simultaneously continued for 1½–2½ hours. To avoid possible hypoxemia in the blood there were 5–10 quiet inspirations between the voluntary inspirations and also 2–3 pauses 5 minutes in duration. In the experiments endeavours were made to minimize fatigue, particularly in view of the following points raised by various researchers: 1) it has been shown that when a skeletal muscle fatigues greater integrated electrical activity is needed to effect a given tension than in a rested muscle. This applies to both isometric (EDWARDS & LIPPOLD 1956) and anisometric contractions (SCHERRER, SAMSON & SOULA 1954); 2) the effect of fatigue on the impulse frequency is not clear: some research workers feel that no change occurs (LINDSLEY 1935, BIGLAND & LIPPOLD 1954 b) have reported a diminution in the frequency (COBB & FORBES 1923), or have observed an increase in the incidence of polyphasic potentials (BUCHTAL, PINELLI & ROSENFALK 1954); 3) exercise has been found to reduce the impulse frequency (JALAVISTO et al. 1938, JALAVISTO, KERANEN & SEPPALA 1939, STEPANOV 1959).

In all the experiments the subjects were in a supine position. Through out series of 6–8 inspirations were effected, with the volume increased at each inspiration (e.g. 0.5, 1.0, 1.5, 2.0, 4.0 l). 10–15 series were registered.

## Statistical methods

The standard deviation ( $s$ ) was calculated by means of the usual formula

$$\hat{s} = \frac{\sqrt{N \sum x^2 - (\sum x)^2}}{N - 1}$$

where  $x$  is a variable and  $N$  the number of observations

The coefficient of linear correlation ( $r$ ) was arrived at from

$$r = \frac{s_{xy}}{s_x s_y},$$

where  $s_{xy}$  is the covariance of variables  $x$  and  $y$ , calculated from

$$s_{xy} = \frac{1}{N - 1} \left[ \sum xy - \frac{\sum x \sum y}{N} \right]$$

The standard deviation of  $x$   $s_x$  was found from

$$s_x = \sqrt{\frac{1}{N - 1} \left[ \sum x^2 - \frac{(\sum x)^2}{N} \right]}$$

and the standard deviation of  $y$   $s_y$  from

$$s_y = \sqrt{\frac{1}{N - 1} \left[ \sum y^2 - \frac{(\sum y)^2}{N} \right]}$$

The regression coefficient was calculated

$$b = \frac{s_{xy}}{s_x^2}$$

and the point at which the regression line meets the  $y$  axis

$$c = \frac{\sum y \sum x^2 - \sum x \sum xy}{N \sum x^2 - (\sum x)^2}$$

The modified standard error of estimate which measures the concentration of the points about the line of regression was calculated from

$$s_{y \cdot x} = s_y \sqrt{1 - r^2} = \sqrt{\frac{N - 1}{N - 2}}$$



The existence of linear stochastic dependence was subjected to a  $t$  test. The coefficient  $t$  was arrived at from

$$t = \frac{r \sqrt{\lambda - 2}}{\sqrt{1 - r^2}}$$

where the number of degrees of freedom is  $\lambda - 2$

The significance of the difference between two regression coefficients from independent regression analyses was subjected to a  $t$  test

$$t_b = \frac{b_1 - b_2}{\sqrt{\frac{s_y^{(1)2}}{(\lambda_1 - 1) s_x^{(1)2}} + \frac{s_y^{(2)2}}{(\lambda_2 - 1) s_x^{(2)2}}}}$$

where the number of degrees of freedom was calculated for simplicity as  $N_1 + N - 2$

The significance of the difference between two constant terms ( $c$ ) was subjected to a  $t$  test

$$t = \frac{c_1 - c_2}{\sqrt{s_{c_1}^2 + s_{c_2}^2}}$$

where

$$s_c = \frac{\sum x \hat{e}_y}{N \sum x^2 - (\sum x)^2}$$

and the number of degrees of freedom is  $N_1 + N$

The mean of the coefficients  $\bar{c}$  corrected for the means of the transformation  $s_{\bar{c}}$

$$\sum_{i=1}^m$$

$$\sum_{i=1}^m$$

where

and

the latter being the  $s^2$

The references are W

## RESULTS

### Experiments under normal conditions

#### *Electromyogram impulse frequency at the end of inspiration and inspiratory muscular work*

The impulse frequency recorded from the external intercostal muscles during a  $1/3$  second interval at the end of inspiration what is known as the impulse frequency of the dynamic phase ( $f_d$ ) bears a linear relationship to the inspiratory muscular work ( $W$ ) during voluntary inspiration within a certain range (Fig. 2) Fig. 2 illustrates the result calculated from 42

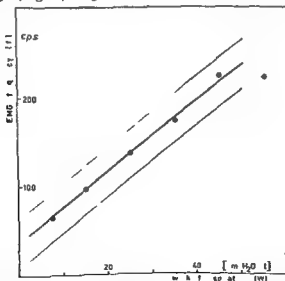


Fig. 2. Electromyogram impulse frequency ( $f_d$ ) at the end of inspiration as a function of inspiratory muscular work ( $W$ ). The points represent the mean of at least 8 values of the variables in columns with a class interval of 10 cm  $H_2O \times l$ . There are 42 values of the variable in the range 0–50 cm  $H_2O \times l$ ; the regression coefficient ( $b_d$ ) is  $4.6$  m  $H_2O^{-1} \times sec^{-1} \times l^{-1}$  and the constant ( $a_d$ ) 33.6 cps; the coefficient of the linear correlation ( $r$ ) 0.90 and the modified standard error of estimate ( $s_y$ )  $\pm 1.9$  cps.

values of the variables within the range  $0-50 \text{ cm H}_2\text{O} \times 1$  the regression coefficient ( $b_d$ ) is  $4.0 \text{ cm H}_2\text{O}^{-1} \times \text{sec}^{-1} \times 1^{-1}$  and the constant ( $c_d$ )  $38 \text{ sec}^{-1}$ , the coefficient of the linear correlation ( $r$ )  $0.90$  the  $t$  test value ( $t$ )  $13.1$  and the modified standard error of estimate ( $\hat{s}_{y \cdot x}$ )  $\pm 27.9 \text{ sec}^{-1}$ . The points represent the mean of at least 5 values of the variables in columns with a class interval of  $10 \text{ cm H}_2\text{O} \times 1$ . The volume of the smallest inspiration included is  $0.58 \text{ l}$  the corresponding muscular work is  $4.4 \text{ cm H}_2\text{O} \times 1$  and the volume of the largest inspiration  $2.74 \text{ l}$  with the representative inspiratory muscular work  $47.9 \text{ cm H}_2\text{O} \times 1$ . The mean of the upper limits of the linear range in the 14 experimental subjects was  $44 \pm 10 \text{ cm H}_2\text{O} \times 1$ . The volumes of the inspirations varied between  $0.4-3.6$  litres. The linearity can be expressed by the equation

$$f_d = b_d W + c_d,$$

where  $b_d$  is the regression coefficient, and  $c_d$  the constant of the line. The arithmetical mean ( $\bar{U}_{b_d}$ ) of the regression coefficients was  $5.1 \pm 2.2 \text{ cm H}_2\text{O}^{-1} \times \text{sec}^{-1} \times 1^{-1}$  and the mean of the constants ( $\bar{U}_{c_d}$ )  $25.3 \pm 30.3 \text{ sec}^{-1}$  (Table 1). The mean of the linear correlation coefficients ( $r$ ) determined by the  $z$  transformation was  $0.90$ . The detailed findings in respect of the individual subjects are listed in Table 1 of the Appendix, which also includes the  $t$  test values ( $t$ ) and the modified standard errors of estimates ( $\hat{s}_{y \cdot x}$ ). The corresponding regression lines are indicated in Fig. 3.

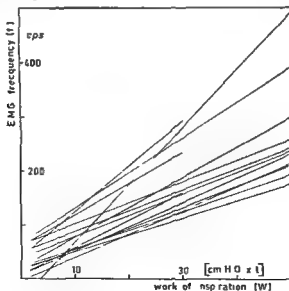


Fig. 3. Electromyogram impulse frequency ( $f_d$ ) at the end of inspiration as a function of the inspiratory muscular work ( $W$ ) from 14 experimental subjects separately.

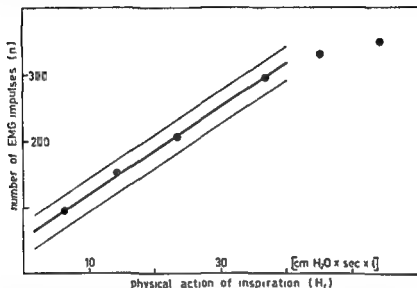
TABLE 1

MEAN VALUES OF REGRESSION COEFFICIENTS ( $M_1$ ) AND CONSTANTS ( $P$ ) DERIVED FROM EQUATIONS  $n = b_n H_i + c$   
 $f_d = b_d f + c_d$   $f = b P + c$  IN RESPECT OF 14 PERSONS where  $n$  is total number of electromyogram impulses  $H_i$ , the first time  
integral of inspiratory muscular work  $f_d$  the electromyogram impulse  $f$  frequency at the end of inspiration  $P$  inspiratory muscular work  $f$   
the electromyogram impulses frequency four seconds after the end of inspiration with the subject holding his breath  $b$   $b_d$  and  $c$  are regression  
coefficients and  $c$   $c_d$  and  $c$  the corresponding constants The mean of the regression coefficients ( $\bar{r}$ ) was determined by means of the trans  
formation

Number of subjects	$N$ number of inspirations	Mean of regression coefficients $\text{cmH}_2\text{O}^{-1} \times 10^{-1}$	Mean of constants $M_1$	Standard deviation $ s $	Mean of coefficients $r$	Number of inspirations $N$	Mean of regression coefficients $\text{cmH}_2\text{O}^{-1} \times 10^{-1}$	Mean of constants $M_2$	Standard deviation $ s $	Mean of coefficients $r$	Number of inspirations $N$	Mean of regression coefficients $\text{cmH}_2\text{O}^{-1} \times 10^{-1}$	Mean of constants $M_2$	Standard deviation $ s $	Mean of coefficients $r$
14	337	7.6	46.3	2.0	0.91	-92	0.1	20.3	2.2	0.90	369	13.1	-38.7	3.7	0.91
														39.0	

# *Total electromyogram impulse number and the first time integral of the inspiratory muscular work*

The total number of the electromyogram ( $n$ ) registered from the external intercostal muscles during inspiration is linearly proportional to the first time integral ( $H_1$ ) of the inspiratory muscular work within a certain range. In Fig. 4, the total impulse number ( $n$ ) of the electro



*Fig. 4* Total number ( $n$ ) of electromyogram impulses as a function of the first time integral ( $H_1$ ) of the inspiratory muscular work. The points represent the mean of at least 6 values of the variables in columns with a class interval of  $10 \text{ cm H}_2\text{O} \times \text{sec} \times \text{l}$ . There are 41 values of the variables in the range  $0-40 \text{ cm H}_2\text{O} \times \text{sec} \times \text{l}$ ; the regression coefficient ( $b$ ) is  $6.7 \text{ cm H}_2\text{O}^{-1} \times \text{sec}^{-1} \times \text{l}^{-1}$  and the constant of the regression line ( $c$ ) 51.1; the coefficient of the linear correlation ( $r$ ) 0.94 and the modified standard error of estimate ( $\hat{\sigma}_y$ )  $\pm 20.4$ .

myogram is given as a function of the first time integral ( $H_1$ ) of the muscular work, the «physical action» (dimension  $\text{cm H}_2\text{O} \times \text{sec} \times \text{l}$  comp. BERGSTROM 1962). The results relate to the same subject as above. The figure contains 41 points in the  $H_1$  range 0 to  $40 \text{ cm H}_2\text{O} \times \text{sec} \times \text{l}$ ; the regression coefficient ( $b$ ) is  $6.7 \text{ cm H}_2\text{O}^{-1} \times \text{sec}^{-1} \times \text{l}^{-1}$  and the constant term ( $c$ ) 51.1; the coefficient of the linear correlation ( $r$ ) is 0.94; the value of the  $t$  test ( $t$ ) 17.2; the modified standard error of estimate ( $\hat{\sigma}_y$ )  $\pm 25.4$ . The points indicate the mean of at least 6 values of the variables in the columns in which the class interval is  $10 \text{ cm H}_2\text{O} \times \text{sec} \times \text{l}$ . The smallest inspiration included is 0.58 l and its physical action ( $H_1$ )  $1.8 \text{ cm H}_2\text{O} \times \text{sec} \times \text{l}$ . The deepest inspiration within the range 0–40

cm H<sub>2</sub>O  $\times$  sec  $\times$  1 is 2.84 l its  $H_i = 39.9$  cm H<sub>2</sub>O  $\times$  sec  $\times$  1. It thus becomes possible to combine the results in the equation

$$n = b H_i + c$$

The arithmetical mean of the regression coefficients ( $M_{b_n}$ ) from the 14 experimental subjects is  $7.6 \pm 2.0$  cm H<sub>2</sub>O<sup>-1</sup>  $\times$  sec<sup>-1</sup>  $\times$  l<sup>-1</sup> and the arithmetical mean of the constants ( $M_c$ ) is  $46.3 \pm 44.3$ . The mean of the linear correlation coefficients ( $r$ ) calculated applying the  $z$  transformation is 0.91 (Table 1). The mean value of the upper limits of the linear ranges in the 14 subjects was  $53 \pm 10$  cm H<sub>2</sub>O  $\times$  sec  $\times$  l. The findings in respect of the individual subjects are given in Table 1 of the Appendix, and the regression lines in Fig. 5.

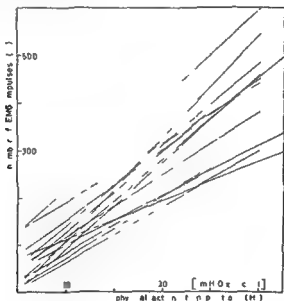


Fig. 5 Total number ( $n$ ) of the electromyogram impulses as a function of the first time integral ( $H_i$ ) of the inspiratory muscular work from 14 experimental subjects separately.

#### *Electromyogram impulse frequency four seconds after the end of inspiration and the alveolar relaxation pressure*

The impulse frequency of the electromyogram, determined four seconds after the end of the inspiration with the subject holding his breath with the glottis open, the so called impulse frequency of the static phase ( $f$ ), bears a linear relationship to the corresponding alveolar

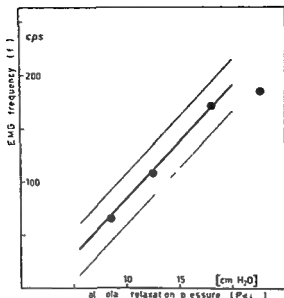


Fig 6 Electromyogram impulse frequency four seconds after the end of inspiration the subject holding his breath ( $f$ ) as a function of the alveolar relaxation pressure ( $P_A$ ) The points represent the mean of at least 6 values of the variables in columns with a class interval of 5 cm  $H_2O$  There are 39 values of the variables within the range 0–20 cm  $H_2O$  the regression coefficient ( $b$ ) =  $10.8 \text{ cm } H_2O^{-1} \times \text{sec}^{-1}$  and the constant of the regression line ( $c$ ) =  $24.5 \text{ cps}$  the coefficient of the linear correlation ( $r$ ) 0.78 and the modified standard error of estimate ( $\hat{s}_y$ )  $\pm 22.0 \text{ cps}$

relaxation pressure ( $P_A$ ) (Fig 6) This again can be expressed by an equation

$$f = b P_A + c$$

where  $b$  is the regression coefficient and  $c$  the constant term Fig 6 relates to the same subjects as above In the figure the regression line is calculated from 39 values of the variables within the range 0–20 cm  $H_2O$  the regression coefficient ( $b$ ) is  $10.8 \text{ cm } H_2O^{-1} \times \text{sec}^{-1}$  and the constant term ( $c$ ) is  $24.5 \text{ sec}^{-1}$  the coefficient of the linear correlation ( $r$ ) 0.78 the value of the  $t$  test ( $t$ ) is 7.6 and the modified standard error of estimate ( $\hat{s}_{y,x}$ )  $\pm 22.0 \text{ sec}^{-1}$  The points are the mean of at least 6 values of the variables in columns with a class interval of 5.0 cm  $H_2O$  The smallest inspiration included is 0.41 l and its alveolar relaxation pressure 5.1 cm  $H_2O$  The deepest inspiration in the range 0–20 cm  $H_2O$  is 2.46 l and its alveolar relaxation pressure 19.6 cm  $H_2O$

The arithmetical mean of the regression coefficients ( $M_b$ ) in respect of the 14 experimental subjects is  $13.1 \pm 3.7 \text{ cm } H_2O^{-1} \times \text{sec}^{-1}$  and that of the constant terms ( $M_c$ ) =  $38.7 \pm 39.6 \text{ sec}^{-1}$  The mean of the linear

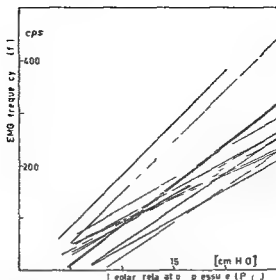


Fig. 7 Electromyogram impulse frequency ( $f$ ) four seconds after the end of inspiration the subject holding his breath as a function of the alveolar relaxation pressure ( $P_A$ ) from 14 experimental subjects separately

correlation coefficient ( $r$ ) ( $z$  transformation) is 0.91 (Table 1). The mean value of the upper limits of the linear range is  $24 \pm 3$  cm H<sub>2</sub>O. The findings with respect to the individual subjects are present in Table 1 of the Appendix and the corresponding regression lines in Fig. 7.

#### *Electromyogram impulse number and the pressure impulse produced by inspiratory muscles*

In Figure 8 the electromyogram impulse number ( $n$ ) during one and the same inspiration is plotted against the corresponding pressure impulse produced by the inspiratory muscles ( $I = \int P_m \cdot \Delta t$  where  $t$  is the duration of the inspiration). It is observable that there exists a linear relationship between these two variables. These can be combined in the equation of the line

$$n = b I + c$$

where  $b$  is the regression coefficient and  $c$  the constant term. The slope of these variable varies in different kinds of inspiration; its steepness is determined at the beginning of inspiration. The arithmetical mean of the coefficient of the linear regression calculated from 100 inspirations recorded from ten different subjects was 0.998. The results



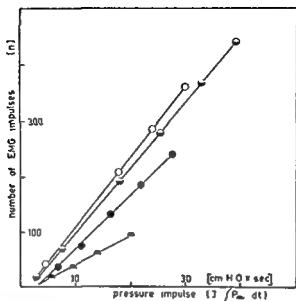


Fig. 8 Number of electromyogram impulses ( $n$ ) as a function of the pressure impulse ( $I$ ) produced by the inspiratory muscles during one and the same inspiration. The figure relates to four different inspirations

obtained from six subjects are given in Tables 2a—3f of the Appendix. This also means that during the deepening of the inspiration the impulse frequency of the electromyogram registered from external intercostal muscles is directly proportional to the pressure produced by the inspiratory muscles ( $P_{ms}$ ) although the slope varies after different inspirations.

### Experiments during $\text{CO}_2$ stimulation

Registration from six experimental subjects were taken before, during and after  $5 \pm 0.4\%$   $\text{CO}_2$  stimulation. The regression lines of the linear correlation, which represent the relationship of the electromyogram impulses ( $n$ ) and the physical action ( $I$ ), are shown in Fig. 9 ( $\circ - \circ - \circ =$  before,  $\bullet - \bullet - \bullet =$  during,  $\ominus - \ominus - \ominus =$  after stimulation). The regression lines are calculated respectively from 37, 11 and 10 inspirations, and the corresponding coefficients of the linear correlation are respectively 0.80, 0.87 and 0.92; the regression coefficients 9.5, 5.1 and 10.8  $\text{cm H}_2\text{O}^{-1} \times \text{sec}^{-1} \times \text{l}^{-1}$ ; the constant terms 124.2, 146.2 and 96.1; and the modified standard errors of the estimates  $\pm 120.7$ ,  $\pm 50.7$  and  $\pm 59.2$  respectively. Figure 9 illustrates that the slope of these variables is less

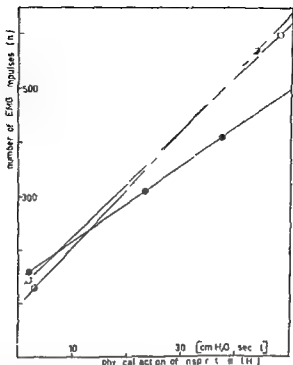


Fig. 2 Total number of electromyogram impulses ( $n$ ) as a function of the first time integral of the inspiratory muscular work ( $H_I$ ) before (O-O-O) during (●-●-●) and after (◐-◐-◐) 50%  $\text{CO}_2$  stimulation

during  $\text{CO}_2$  stimulation than before and after it. Table 2 contains the arithmetical means of the regression coefficients ( $M_{b_i}$ ) and of the constant terms ( $M$ ) and their standard deviations ( $\hat{s}$ ) before (A) during (B) and after (C) stimulation. There are further the means of the coefficients of the linear correlations ( $r$ ) calculated by the  $z$  transformation which are 0.92, 0.88 and 0.91 respectively. Table 2 also gives the arithmetical means of the regression coefficients of equations  $f_d = f(H)$  and  $f = f(P_{(i)})$  and the corresponding mean of the coefficients of the linear correlations ( $r$ ) ( $z$  transformation). The linearity is evident in these equations; in the first case the values of  $r$  are 0.94, 0.90 and 0.93 respectively and in the second 0.92, 0.87 and 0.94.

It is observable from Fig. 2, Table 2 and Table 2 of the Appendix which contains the results in respect of individual subjects that the slope between the number of electromyogram impulses and the physical action is less during  $\text{CO}_2$  stimulation than before and after it, although the  $t$  test indicates a statistical significant difference ( $p < 0.01$ ) in only

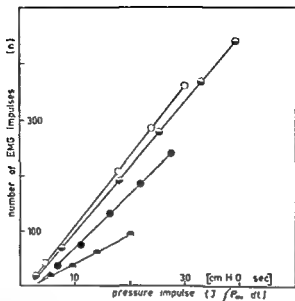


Fig. 8 Number of electromyogram impulses ( $n$ ) as a function of the pressure impulse ( $I$ ) produced by the inspiratory muscles during one and the same inspiration. The figure relates to four different inspirations.

obtained from six subjects are given in Tables 3 a—3 f of the appendix. This also means that during the deepening of the inspiration the impulse frequency of the electromyogram registered from external intercostal muscles is directly proportional to the pressure produced by the inspiratory muscles ( $P_m$ ) although the slope varies after different inspirations.

### Experiments during $\text{CO}_2$ stimulation

Registration from six experimental subjects were taken before, during and after  $5 \pm 0.4\%$   $\text{CO}_2$  stimulation. The regression lines of the linear correlation which represent the relationship of the electromyogram impulses ( $n$ ) and the physical action ( $H$ ) are shown in Fig. 9 (o—o—o = before, ●—●—● = during, ◐—◐—◐ = after stimulation). The regression lines are calculated respectively from 37.8 and 10 inspirations, and the corresponding coefficients of the linear correlation are respectively 0.80, 0.87 and 0.92, the regression coefficients 9.5, 5.1 and 10.8  $\text{cm H}_2\text{O}^{-1} \times \text{sec}^{-1} \times \text{l}^{-1}$ , the constant terms 124.2, 146.2 and 96.8 and the modified standard errors of the estimates  $\pm 120.7$ ,  $\pm 50.7$  and  $\pm 59.2$  respectively. Figure 9 illustrates that the slope of these variables is less

one case in the course of transfer from A state to B state ( $t_4 = 2.75$ ) and in three cases from B state to C state ( $t_4 = 9.75, 3.21, 3.02$ ) (refer to Table 2 in the Appendix,  $t_4$ ). Moreover the constant term ( $c$ ) is always numerically greater during CO stimulation than before and after it although a statistical significant difference is apparent twice only ( $t = 3.18$  and  $3.36$ ). In three instances the regression coefficient ( $b_d$ ) between the impulse frequency of the dynamic phase ( $f_d$ ) and the inspiratory muscular work (II) is significantly less during CO stimulation than before it ( $t_4 = 3.90, 3.35$  and  $3.32$ ) and in three instances less than after stimulation ( $t_4 = 4.14, 5.09$  and  $5.60$ ). With regard to constant ( $c_d$ ) there is one statistical significant difference ( $t = 2.88$ ).

To judge from the regression coefficient ( $b$ ) between the electromyogram impulse frequency of the static phase ( $f$ ) and the alveolar relaxation pressure ( $P_{11}$ ) in none of the cases is there any statistical significant difference this applies also to the corresponding constant ( $c$ ). The findings in respect of the individual subjects are presented in Table 2 of the Appendix.

During CO stimulation the linear correlation between the electromyogram impulse number ( $n$ ) and the corresponding pressure impulse ( $I$ ) remains. The arithmetic mean of the coefficients of the linear correlations calculated from 60 inspirations recorded from six subjects was 0.998. The findings in respect of the individual subjects are contained in Table 3a—3f in the Appendix along with the results of post stimulation registration.

### Comparison of the electromyograms recorded with surface electrodes and needle electrodes

Fig. 10 shows the impulse number ( $n$ ) of the electromyogram taken from 48 inspirations recorded with concentric needle electrodes plotted against the electromyogram impulse number ( $n$ ) recorded simultaneously with surface electrodes. These variables are clearly in linear correlation. The regression coefficient is 0.41 and the coefficient of linear correlation 0.98.

The regression coefficient is the same 0.41 on comparison with the impulse frequency of the electromyogram registered at the end of the inspiration. The coefficient of linear correlation is 0.96. The experiments were concerned with six experimental subjects and the correlation coefficient ( $r$ ) varied between 0.95—0.99 ( $p < 0.001$ ).

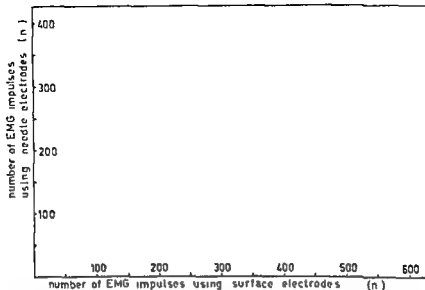


Fig 10 Electromyogram impulse number ( $n$ ) recorded with concentric needle electrodes from intercostal muscles as a function of the electromyogram impulse number ( $n$ ) recorded with surface electrodes from the same muscles. The regression coefficient is 0.41 and the coefficient of linear correlation ( $r$ ) 0.98 calculated from 48 values of variables

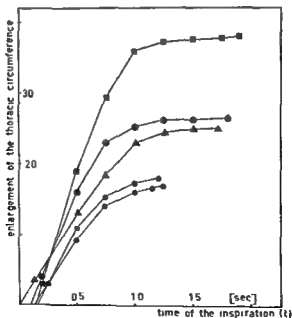


Fig 11 Enlargement of the thoracic cage as a function of time during five different inspirations. The unit of enlargement is arbitrary

## Movements of the thoracic cage in inspiration

Fig. 11 illustrates a typical example of the enlargement of the thoracic cage during inspiration as a function of time. It is evident that the thoracic cage is enlarged in different ways during different parts of inspiration, and furthermore that the velocity at the beginning is rather rapid throughout the same inspiration, and becomes slower at its end.

## DISCUSSION

Gradation of the voluntary contraction of the skeletal muscle in man is based upon recruitment of the motoneurons their action frequency, and action time (ADRIAN & BROOK 1929 SMITH 1934 LINDSLEY 1935 GILSON & MILLS 1941 TOKIZANE, KAWATA & TOKIZANE 1952, BIGLAND & LIPPOLD 1954 b) in regard to the skeletal muscles this has been demonstrated by TOKIZANE et al (1952) for the intercostal muscles specifically. Similar gradation has been found in animals both in the intercostal muscles (BRONK & FERGUSON 1935, GESELL ATKINSON & BROWN 1941 GESELL HUNTER & LILLIE 1949) and in the diaphragm (GESELL et al 1941 PITTS 1942) BERGSTROM (1958 1959 b) reduced these three gradating factors to one alone, the number of action potentials of the set of motoneurons involved in the muscle contraction.

Nevertheless it has been shown that the electromyogram impulses are equivalent to the nerve impulses fired into the muscle during the contraction (DITTLER 1910 DITTLER & GARTEN 1912 GASSER & NEWCOMER 1921). Consequently the electromyogram impulses also represent the corresponding nerve impulses.

It is impossible to record the total electrical activity of the intercostal muscles. All that can be done is to take a sample from the total electrical activity of an electromyographical registration as was effected by BERGSTROM & KERTTULA (1961). It can be assumed that the sample taken by a non selective technique represents the total electrical activity. This means that these two bear a linear mutual relationship. This is also supported by the linear relationship of the electromyogram activities recorded simultaneously by means of surface and concentric needle electrodes (Fig. 10). Furthermore, BIGLAND & LIPPOLD (1954 b), on comparing surface electrodes with concentric needle electrodes observed that the integrated electrical activities from the extensor digitorum communis had a linear relationship at low activity levels in isometric contraction. They concluded that addition and subtraction occur in

such a way that the final output remains constant DELHEZ & PETIT (1961) have shown that both methods of detection provide similar results. They recorded electromyograms from human abdominal muscles from m sternocleidomastoideus and m scalenus and also from m trapetzius and m pectoralis major but pointed out a marked variability in the individual values for activity. ANDERSSON & LINDSLEY (1935) have demonstrated that the external intercostal muscles of two sides of the thorax in normal and unilateral pneumectomized cats are activated equally both in regard to the number of units involved and the average frequency of the responses in each. The single motor unit technique was applied. GASSER & NEWCOMER (1921) recorded impulses from the right phrenic nerve and the left portion of the diaphragm observed that the discharges were symmetrical and decided that a common centre must regulate impulsion on both sides. BOYD & BASMAJAN (1963) implanted multiple clip electrodes in the diaphragm of rabbits at open operation, and found no obvious general pattern of difference in the electric activity between various muscular slips of the same rabbit's diaphragm. TAYLOR (1960) took simultaneous recordings from human diaphragm through a needle electrode at the costal margin and through oesophageal electrodes and discovered no real difference in the timing of the activity detected in the two sites.

When the size and position of the electrodes and other experimental conditions are kept constant it has been assumed in the present study that with a non selective technique the electromyogram represents the total motor discharge fired by the nervous system into the inspiratory muscles during an inspiratory cycle as supposed by BERGSTROM & KERTTULA (1961) in regard to the intercostal muscles of rats. This implies that at the same time as reference is made to the electrical and mechanical activity of the intercostal muscles there is reference to the relation between the electrical activity of the intercostal nerves and the mechanical activity of the inspiratory muscles.

On comparison of the impulse frequency of the electromyogram recorded from external intercostal muscles at the end of inspiration and the inspiratory muscular work done in inspiration frequency ( $f_d$ ) was found to have a linear relationship to the work ( $W$ ) (Fig. 2). The results given above have also shown that the total number of impulses counted from the electromyogram sample during inspiration is directly proportional to the first time integral of the inspiratory muscular work ( $H_1$ ) the so called physical action (dimension  $\text{cm H}_2\text{O} \times \text{sec} \times l$  cf BERGSTROM 1962) (Fig. 4). These results are in accord with findings in respect to the activity in the intercostal muscles of rats where also the electromyogram



impulse frequency taken at the end of inspiration is linearly proportional to the relative inspiratory work determined from the amplitude of the volume curve and from changes in the intra pleural pressure. In these experiments it was further demonstrable that the total number of the action potentials determined from the electromyogram sample rose proportionally to the time integral of the respiratory work (BERGSTROM & KEPTTULA 1961)

In the main the same finding was made by BERGSTROM in his experiments with voluntary finger muscles of the human being the results indicate that the impulse frequency of the electromyogram is directly proportional to the degree of muscle shortening at different loadings of the contractions the amount of work produced by unloaded contraction and also to the estimated muscular work necessary to attain the degree of abduction (1959 c). He has further demonstrated that the number of electromyogram impulses bears a linear relationship to the physical action (1959 b).

From the results outlined above there can be written

$$n = b \int H_1 + c$$

where  $n$  is the total number of action potentials  $H_1$  the first time integral of the work ( $H_1 = \int H \times dt$ )  $b$  is the regression coefficient, and  $c$  the constant. The right side of the equation is thus a function of time as is also magnitude  $n$  which involves the three gradating factors the number of the recruited motor units their action frequency and action time. During inspiration the retraction force of the respiratory system tries to return the system to the functional respiratory level. This entails that the longer the inspiration takes the greater the number of action potentials becomes during a given voluntary inspiration although the inspiratory work is not increasing with time. This implies that the results reported by DELHEZ et al (1965) and DELHEZ PETIT & BOTTIN (1965), which indicate that the integrated electromyogram activity of the human diaphragm is directly proportional to inspiratory work are valid for these reasons only when the duration of inspirations is constant since the integrated electrical activity is a function of time but the work is not.

Earlier it was shown that the electrical activity of the skeletal muscle bears a linear relationship to the muscle tension both in isometric (JALAVISTO et al 1939 LIPPOLD 1952) and in anisometric contraction (BIGLAND & LIPPOLD 1954 a). In principle this is also apparent in the finding that the impulse frequency of the electromyogram determined four seconds after the end of the inspiration with the subject holding his breath with the glottis open the impulse frequency of the static

phase ( $f$ ) is in linear relation to the corresponding alveolar relaxation pressure ( $P_{al}$ ) (Fig. 6). Here the contraction of the intercostal muscle is anisometric, but the velocity is zero. The four second interval after the end of the inspiration was chosen in view of earlier experiments (VILJANEK et al 1965). In that phase the electromyogram activity of the intercostal muscles has reached almost a constant level after the inspiration; moreover the subject can quite easily hold his breath for such a period.

It is interesting to observe that during one and the same inspiration the electromyogram impulse number ( $n$ ) is directly proportional to the force impulse of the inspiratory muscles, here expressed as the pressure impulse ( $I = \int P_m \times dt$ ) (Fig. 8). This means that during inspiration the electromyogram impulse = frequency bears a linear relation to the pressure produced by the inspiratory muscles ( $P_m$ ). The slope between the impulse number ( $n$ ) and the pressure impulse ( $I$ ) varies in different types of inspiration but the linear regression still remains. If a start is made from the linear correlation of the electrical activity of the intercostal muscles and the pressure produced by the inspiratory muscles ( $P_m$ ) it can be concluded that during the whole inspiration the relative quantity of the inspiratory muscles remains on a constant level with the total inspiratory muscular force ( $P_m$ ). It is likely that in different inspirations variations occur in the proportion of the intercostal muscles which form the total inspiratory muscular force although it remains constant within one and the same inspiration. It also seems that at the beginning of each inspiration the respiratory centres determine which muscles will take part in the inspiration and the extent of such participation (VILJANEK 1967 b). It should be pointed out that in deep inspirations in which the physical action is great the linearity between the total electromyogram impulse number ( $n$ ) and the physical action ( $H$ ) no longer applies (Fig. 4). In each of these inspirations the electromyogram impulse number rises in a linear relation to the corresponding pressure impulse except as regards very forced inspirations.

Accordingly in each inspiration with the exclusion of those which are very forced in nature the electromyogram impulse frequency is directly proportional to the pressure produced by the inspiratory muscles ( $P_m$ ). Nevertheless at the end of the inspiration the electromyogram impulse frequency of the external intercostal muscle also bears a linear relation ship to the inspiratory muscular work (Fig. 2). This means that at the end of the inspirations the muscular force expressed as pressure ( $P_{ma}$ ) and the muscular work ( $W$ ) are in direct proportion according to the formal equation

$$P_m = a \times V = a \times \int P_m \times dV,$$

where  $a$  is a constant  $V$  = the tidal volume. Consequently the force and the distance, and thus the pressure ( $P_m$ ) and the volume ( $V$ ) must have a logarithmic relation when other factors are constant. Further, the impulse frequency of the intercostal muscle electromyogram at the end of inspiration ( $f_d$ ) and the tidal volume ( $V$ ), have a logarithmic relation when the other factors remain constant (VILJANEN 1966). This can be expressed by the equation

$$\ln f_d = k_1 + k \times V,$$

where  $k_1$  and  $k$  are constants.

Possibly the electromyogram impulse frequency during the whole inspiration would be in logarithmic relationship to the increase in tidal volume were it not for the increased firing of motor impulses from the muscular spindle systems stimulated by the flow resistive and inertial muscle tension, which are zero at the end of the inspiration. The firing of motor impulses from the spindle systems has been demonstrated by CRITCHLOW & von EULER (1962) in cats.

Furthermore it seems that the respiratory centres are programmed to find a determined end state in the inspiratory volume during each inspiration, reached also by the respiratory system if the peripheral conditions stay constant. It might be that the pursuit of a given volume results from the sending of a determined nervous impulse train to the inspiratory muscles and that the physical action of the inspiratory muscle is determined by this means. The number of impulses might then be one explanation for the demand for tidal volume in CAMPBELL & HOWELL's (1962) hypothesis on the nervous regulation of respiration. A possible explanation of the findings of CAMPBELL & his co-workers (1961a) and CAMPBELL, DINNICK & HOWELL (1961) that a sudden increase of the inspiratory load is followed by a diminution in the tidal volume and overcome only progressively is that after the delivery of the impulse train the nervous centres can no longer correct the impulse number. Moreover there exists the possibility that a sudden reduction in the load involves a corresponding deepening of the tidal volume. In other words, with a given impulse number it is possible to release a certain amount of energy. This is in accord with the findings of PAMOS & ARJONA (1956) that the electrical activity of the diaphragm bears a linear relationship to the oxygen consumption. It could be said that from 'experience' (plus additional other orders) the inspiratory centres know how many

nervous impulses are needed for an adequate tidal volume. Under normal conditions this amount results in attainment of the tidal volume required. Further, it also seems possible that the above is an example of a more general central nervous system control of the muscular function suggested by BERGSTROM (1958).

If the velocity of the respiratory system during one and the same inspiration is constant as it appears to be over a wide range at least (Fig. 11) the electromyogram impulse number registered from the external intercostal muscle during one inspiration is in a linear relationship to the inspiratory muscular work since the electromyogram impulse number and the pressure impulse ( $I$ ) bear a linear relation as well (Fig. 8). At the end of the inspiration, this no longer applies, since there the increase in the volume is nearing zero as is consequently the inspiratory muscular work as well although electromyogram impulses are continuously discharged in order to balance the retraction force of the lung.

During the course of  $5.0 \pm 0.4\%$   $\text{CO}_2$  stimulation these relationships between the electrical and mechanical activity are valid. The results obtained are in agreement with the findings of BERGSTROM & KERTTULA (1961) in regard to the intercostal muscles of the rat: they demonstrated that during  $\text{CO}_2$  stimulation the linear relations still existed between the electromyogram impulse number registered from the external intercostal muscle of the rat during inspiration and the physical action as well as between the electromyogram impulse frequency at the end of the inspiration and the relative inspiratory work. It is evident that in each experiment the slope of the linear regression between the number of the electromyogram impulses and the physical action is less during  $\text{CO}_2$  stimulation than before and after it (Table 2, Table 2 of the Appendix). No more than a few experiments were conducted with  $\text{CO}_2$  stimulation, in view of the discomfort which arises if  $5\%$   $\text{CO}_2$  air is breathed for an extended period. This may explain the existence of statistically significant differences in some cases only. A similar tendency was also apparent in the relationship between the impulse frequency of the dynamic state ( $f_d$ ) and the inspiratory muscular work ( $W$ ) (Table 1 and Table 2 of the Appendix). During the  $\text{CO}_2$  stimulation larger EMG impulse values were also registered at the beginning of inspiration although the differences were not statistically significant (Table 2, Fig. 9).

It is interesting to note the diminution of the correlation coefficient in the  $\text{CO}_2$  stimulation experiments: this might be attributable to the rising end-tidal  $\text{CO}_2$  tension increasing the work output (MILIC-EMILI & TYLER 1963) although some other factors may be implicated as well e.g. a smaller proportion of the intercostals being recruited.

All the present recordings were made from human intercostal muscles. The studies have been extended to the human diaphragm. Preliminary findings indicate the likelihood of a similar relationship existing between the electrical and mechanical activities in the human diaphragm (VILJANEN 1967 a).

## SUMMARY

1 A comparison has been made between the electromyographically registered activity of the intercostal muscles in man during voluntary inspiration and the corresponding mechanical activity of the inspiratory muscles i.e. the pressure and the pressure impulse produced by inspiratory muscles, the inspiratory muscular work and the first time integral of the inspiratory muscular work calculated from the alveolar relaxation pressure, oesophageal pressure and tidal volume. The experiments were conducted both under normal conditions and during CO<sub>2</sub> stimulation.

2 The total electromyogram impulse number during the course of inspiration bears a linear relationship to the first time integral of the inspiratory muscular work, the so called «physical action». However this linearity did not apply to either very forced or deep inspiratory excursions.

3 The electromyogram impulse frequency registered during a period of 1/3 second at the end of inspiration bears a linear relationship to the inspiratory muscular work.

4 The electromyogram impulse frequency registered within an interval of 1/3 second four seconds after the end of inspiration and with the subject holding his breath with open glottis is directly proportional to the alveolar relaxation pressure.

■ During one and the same inspiration the electromyogram impulse number is directly proportional to the pressure impulse. This means that during one inspiration the electromyogram impulse frequency registered from the external intercostal muscle bears a linear relationship to the inspiratory muscular force ( $P_m$ ). The regression coefficient or the slope of this relationship is determined at the beginning of inspiration and varies between different inspirations.

■ These linear relationships were also observed during CO<sub>2</sub> stimulation.

7 Evidence was obtained of a change of the slope in the electro-mechanical relationship during  $5 \pm 0.4\%$  CO<sub>2</sub> air stimulation.

8 On the basis of the results obtained, it is suggested that the respiratory centres determine in advance which inspiratory muscles are to participate in the inspiratory excursion and which is the strength of contraction of the muscles. The electromyogram impulse frequency at the end of inspiration during stable conditions is in a logarithmic relation to the tidal volume. The central nervous system thus seems to program each inspiration to reach a predetermined end state.

## REFERENCES

- ADRIAN E D and D W BROOK The discharge of impulses in motor nerve fibres Part I  
Impulses in single fibres of the phrenic nerve *J Physiol (Lond)* 1948 66 81-101
- — — The discharge of impulses in motor nerve fibres *J Physiol (Lond)* 1949  
67 119-151
- ACOSTONI E A graphical analysis of thoracoabdominal mechanics during the breathing cycle *J appl Physiol* 1961 16 1039-1039
- — — G SantAMBROGIO and H DEL PORTILLO GARRASCO Electromyography of the diaphragm in man and transdiaphragmatic pressure *J appl Physiol* 1960 15 1093-1097
- ANDERSON F M and D H LANDSLEY Action potentials from intercostal muscles before and after unilateral pneumotomy *J Lab clin Med* 1935 20 623-678
- ATKINSON A K, P C BROWN and R GESSLE The nervous gradation of muscular contraction *Amer J Physiol* 1940 129 P303
- ATTINGER E O, R G MONROE and M S SEGAL Mechanics of breathing in different body positions I In normal subjects *J clin Invest* 1936 35 904-911
- BAYER H und C FLECHTENMACHER Ermüdung und Aktionsstromspannung bei der isometrischen Muskelkontraktion des Menschen *Arbeitsphysiologie* 1930 14 261-270
- BERGSTRÖM R M Über die Anzahl der motorischen Impulse (die Periodenzahl) des Electromyogramms als quantitativer Ausdruck der voluntären zentralen Kontrolle der Muskelaktion *Acta physiol scand* 1938 43 349-358
- — — The relation between the number of impulses and the integrated electric activity in electromyogram *Acta physiol sc* and 1939 a 45 91-101
- — — The kinetic energy produced by voluntarily controlled muscle action and the frequency of the motor discharge *Acta physiol scand* 1939 b 47 179-190
- — — The mechanical work produced by voluntarily controlled muscle action and the frequency of motor discharge *Acta physiol scand* 1939 c 47 191-193
- — — The relation between the integrated kinetic energy and the number of action potentials in the electromyogram during voluntary muscle contraction *Ann Acad Sci fenn* 4 5 1962 93
- — — and Y KERTTULÄ On the neural control of breathing as studied by electromyography of the intercostal muscles of the rat *Ann Acad Sci fenn A* 5 1961 79
- BIGLAND II and O C J LIFFOLD The relation between force velocity and integrated electrical activity in human muscles *J Physiol (Lond)* 1954 a 123 214-224
- — — — — Motor unit activity in the voluntary contraction of human muscle *J Physiol (Lond)* 1954 b 125 321-335



- BISHOP G H and J J BROUWER The site of action of botulinus toxin *Amer J Physiol* 1936 11, 393-401
- BOYD W H and J V BASMAJIAN Electromyography of the diaphragm in rabbits *Amer J Physiol* 1963 204 943-948
- BRONI D W and L K FERGUSON The nervous control of intercostal respiration *Amer J Physiol* 1935 110 700-70
- BUCHTAL F P PINELLI and P ROSENBLAT Action potential parameters in normal human muscle and their physiological determinants *Acta physiol scand* 1964 3, 219-229
- BUTLER J and H SMITH Pressure-volume relationships of the chest in the completely relaxed anaesthetized patient *Clin Sci* 1957 16 155-166
- BUYTENDIJK H J Oesophagusdruck en longelasticiteit I Groningen Oppenheim 1949
- et M FAD J M B McILROY A J SELFRISTONE and H C KRITTE Measurements of intraoesophageal pressure *J appl Physiol* 1955 7 491-495
- CAMPBELL J M An electromyographic examination of the role of the intercostal muscles in breathing in man *J Physiol (Lond)* 1955a 101 1-26
- - - The respiratory muscles and mechanics of breathing. Lloyd-Luke London 1958
- - - C J DICKINSON O I DICKINSON and J B L HOWELL The immediate effects of added elastic loads on breathing *J Physiol (Lond)* 1961a 105 4-16
- - - O P DICKINSON and J B L HOWELL The immediate effects of elastic loads on the breathing of man *J Physiol (Lond)* 1961 106 260-273
- - - and J H GREEN The expiratory function of abdominal muscles in man *J Physiol (Lond)* 1953a 100 401-418
- - - - - The variations in intra-abdominal pressure and the activity of the abdominal muscles during breathing a study in man *J Physiol (Lond)* 1953b 102 282-290
- - - and J B L HOWELL Proprioceptive control of breathing In De Reuck A V S O'Connor Maevie (Eds) Ciba Foundation Symposium on Pulmonary Structure and Function pp 29-45 J & A Churchill London 1962
- CHEVALLIER M H D Patterns of respiratory discharge in the cat and rat *J Physiol (Lond)* 1957 133 33-34 P
- CHEVNIACI R M Physical properties of the lung in chronic obstructive pulmonary emphysema *J clin Invest* 1956 35 394-404
- CHRISTIE R V Dynamics in relation to the viscoelastic properties of the lung *Proc Roy Soc Med* 1953 46 381-386
- CLOSE J R E D NICKEL and F N TODD Motor unit action - potential counts Their significance in isometric and isotonic contractions *Bone Jt Surg* 1960 42A 1207-1211
- COUD S and A FORBES Electromyographic studies of muscular fatigue in man *Amer J Physiol* 1953 65 231-251
- CRANE M G D A HAMILTON and J E VEFFELDT A plastic balloon for recording intraoesophageal pressures *J appl Physiol* 1956 8 585-586
- CRITCHLOW V and C von EULER Rhythmic control of intercostal muscle spindles *Experimentia (Basel)* 1962 18 426-427
- DEFARES J G and P G DOYLEBEN Relationship between frequency dependent compliance and unequal ventilation *J appl Physiol* 1960 15 166-169
- DELHEZ L et J M PETIT Examen comparatif des modalités d'activité électrique des muscles respiratoires au moyen d'électrodes concentriques et d'électrodes de surface chez l'homme *Acta tuber pneumol belg* 1961 11 63-67

- DELHYZ L J M PETIT et R BOTTIN Activité électrique intégrée du diaphragme fonction du travail inspiratoire *J Physiol (Paris)* 1963 57 5 593-599
- — — R SIEFF et R BOTTIN Particularités méthodologiques et intérêt de l'électromyographie intégrée des piliers du diaphragme *Rev Electrodiagn therap* 1963 3 303-317
- DITTLER P Über die Aktionsströme des Nervus phrenicus bei natürlicher Innervation *Pflügers Arch ges Physiol* 1910 131 281-288
- — und H GARTEN Die zeitliche Folge der Aktionsströme in phrenicus und Zwerchfell bei der natürlichen Innervation *Biol* 1912 29 420-420
- DORNEHORST A C and G L LEATHART A method of assessing the mechanical properties of lungs and air passages *Lancet* 1952 263 109-111
- DRAPER M H P LADEFOGED and D WHITTERIDGE Expiratory muscles involved in speech *J Physiol (Lond)* 1957 138 17-18 P
- EDWARDS R G and O C J LIPPOLD The relation between force and integrated electrical activity in fatigued muscle *J Physiol (Lond)* 1956 13 677-681
- EHRNER L Lung compliance and respiratory resistance determined from time marked oesophageal pressure tidal volume curves and their relation to some other tests of lung function *Acta med scand* 1960 suppl 353 1-60
- FERRIS B G J MEAD Jr and N R FRANK Effect of body position on oesophageal pressure and measurement of pulmonary compliance *J appl Physiol* 1950 14 521-524
- FINE B R H NOAI and H A DUNCAN Effect of air flow resistance on ventilation and respiratory muscle activity *Amer med Ass* 1958 168 2245-2249
- FLEISCH A Der Pneumotachograph ein Apparat zur Geschwindigkeitsregistrierung der Atemluft *Pflügers Arch ges Physiol* 1909 713-714
- — — Le Pneumotachograph *Helv physiol pharmacol Acta* 1906 14 363-368
- FRAZER D L W W STEAD R V EBERT R J LUBIN and H H WELLS The measurement of intraoesophageal pressure and its relationship to intrathoracic pressure *J Lab clin Med* 1952 40 664-673
- GABER H S The analysis of individual waves in the phrenic electroneurogram *Amer J Physiol* 1958 85 569-576
- — — and H S NEWCOMER Action currents in the phrenic nerve *Amer J Physiol* 1951 57 1-26
- GEIGY A Jr Documents Geigy Wissenschaftliche Tabellen Ed R Diem Basel 6 Auflage 1960
- GESELL R Fusillade patterns of inspiratory and expiratory muscles and their effects on the respiratory act *Amer J Physiol* 1936 116 28-338
- — — and A K ATKINSON A comparison of motor integration in the mouse rat rabbit dog and horse *Amer J Physiol* 1943 139 745-755
- — — and R G PROWSE The gradation of the intensity of inspiratory contraction *Amer J Physiol* 1941 131 659-673
- — — J HUNTER and R H LILLIE Electrical and functional activity of motor neurons *Amer J Physiol* 1949 159 17-28
- GILL P K The effects of end tidal  $CO_2$  on the discharge of individual phrenic motor neurones *J Physiol (Lond)* 1963 168 239-257
- GILSON A S Jr and W B MILLS Activities of single motor units in man during slight voluntary efforts *Amer J Physiol* 1941 133 658-669
- GREEN J H and J B L HOWELL The correlation of intercostal muscle activity with respiratory air flow in conscious human subjects *J Physiol (Lond)* 1959 149 471-476

- BISHOP C H and J J BROUEN-BRENNER The site of action of botulinus toxin *Amer J Physiol* 1936 117 393-404
- BOYD W H and J V BASILIAN Electromyography of the diaphragm in rabbits *Amer J Physiol* 1963 204 943-948
- BROOK D W and L K FROLOSON The nervous control of intercostal respiration *Amer J Physiol* 1935 110 700-707
- BUCHTAL F P PINELLI and P ROSENFELT Action potential parameters in normal human muscle and their physiological determinants *Acta physiol scand* 1964 30 219-229
- BUTLER J and B SMITH Pressure-volume relationships of the chest in the completely relaxed anaesthetized patient *Clin Sci* 1957 16 137-146
- BUITENDIJK H J Oesophagusdruck en longelasticiteit I Groningen Oppenheim 1949 of MEAD J M B McILROY A J BELVERSTON and B C KRIPPE Measurements of intraoesophageal pressure *J appl Physiol* 1955 7 401-405
- CAMPBELL L J M An electromyographic examination of the role of the intercostal muscles in breathing in man *J Physiol (Lond)* 1955a 120 1-26
- The respiratory muscles and mechanics of breathing Lloyd Luke London 1959
- C J DICKINSON O I DINWICK and J B L HOWELL The immediate effects of added elastic loads on breathing *J Physiol (Lond)* 1961a 150 42 P
- O P DINWICK and J B L HOWELL The immediate effects of elastic loads on the breathing of man *J Physiol (Lond)* 1961 146 260-273
- and J H GRIFEN The expiratory function of abdominal muscles in man *J Physiol (Lond)* 1953a 120 400-419
- — The variations in intra abdominal pressure and the activity of the abdominal muscles during breathing a study in man *J Physiol (Lond)* 1953b 122 282-290
- and J B L HOWELL Proprioceptive control of breathing In De Reuck A V S O Connor Maevs (Eds) Ciba Foundation Symposium on Pulmonary Structure and Function pp 29-45 J & A Churchill London 1960
- CHEVREUILS M H D Patterns of respiratory discharge in the cat and rat *J Physiol (Lond)* 1957 138 33-34 P
- CHERNIACK R M Physical properties of the lung in chronic obstructive pulmonary emphysema *J clin Invest* 1956 35 394-404
- CHESTNUT R V Dyspnoea in relation to the visco-elastic properties of the lung *Proc roy Soc Med* 1953 46 381-386
- CLOSE J R E D WICKEL and F N TODD Motor unit action — potential counts Their significance in isometric and isotonic contractions *Bone Jt Surg* 1960 42A 1207-1211
- COHEN S and A FORBES Electromyographic studies of muscular fatigue in man *Amer J Physiol* 1953 85 231-251
- CRANE M G D A HAMILTON and J E AFFELDT A plastic balloon for recording intraoesophageal pressures *J appl Physiol* 1956 8 555-556
- CRITCHLOW V and C VON EULER Rhythmic control of intercostal muscle spindles *Experimentia (Basel)* 1962 18 426-427
- DEFARES J G and P G DONLEBEN Relationship between frequency dependent compliance and unequal ventilation *J appl Physiol* 1960 15 166-169
- DELHEZ L et J M PETIT Examen comparatif des modalités d'activité électrique des muscles respiratoires au moyen d'électrodes concentriques et d'électrodes de surface chez l'homme *Acta tuber pneumol belg* 1961 11 623-627

1. A. H. W. O. FEY and H. RAHN: Mechanics of breathing in man. *J appl Physiol* 1950 2: 592-607
2. C. B. MCKERROW, R. A. BARTLETT, J. MEAD, M. H. McILROY, N. J. SELVERSTONE and E. P. RADFORD JR.: Mechanical factors in distribution of pulmonary ventilation. *J appl Physiol* 1956 8: 427-443
3. J. M. L. DELHEZ and J. TROQUET: Aspects actuels de la mécanique ventilatoire. *J Physiol (Paris)* 1965 57: 7-113
4. — and J. MILIC-EMILI: Measurement of endoesophageal pressure. *J appl Physiol* 1958 13: 481-485
5. — and L. DELHEZ: Role of the diaphragm in breathing in conscious normal man: an electromyographic study. *J appl Physiol* 1960 16: 1101-1106
6. R. F. FITTS: The function of components of the respiratory complex. *J Neurophysiol* 1949 5: 403-413
7. H. A. B. OTIS, L. E. CHADWICK and W. O. FEY: The pressure-volume diagram of the thorax and lung. *Amer J Physiol* 1946 146: 161-178
8. G. J. RAMOS and P. ARJONA: Oxygen consumption of the breathing processes. *Amer J Physiol* 1956 184: 359-364
9. G. H. BEHN, W. GEBHARDT, P. H. ROSSLER and A. BUTLMAN: Atemmechanischen Untersuchungen am Lungenmodell bei Lungengesunden und bei Patienten mit obstructivem Emphysem. *Schweiz med Wochr* 1957 87: 374-381
10. P. JULIAN: L'étude des activités des centres nerveux par l'exploration oscillographique de leurs voies efférentes. 1. *Arch int Physiol* 1937 44: 351-356
11. —: L'étude des activités des centres nerveux par l'exploration oscillographique de leurs voies efférentes. 2. *Arch int Physiol* 1937 44: 387-404
12. F. POHRER: Der Strömungswiderstand in den menschlichen Atemwegen und der Einfluss der unregelmässigen Verzweigung des Bronchialsystems auf den Atemungsverlauf in verschiedenen Lungenbezirken. *Pflügers Arch ges Physiol* 1915 162: 220-300
13. —: Der Zusammenhang der Atemkräfte und ihre Abhängigkeit vom Dehnungszustand der Atemungsorgane. *Pflügers Arch ges Physiol* 1916 163: 419-444
14. P. H. H. J. NIEFORENT, H. PIPBERGER and R. HALM: Elektromyographische Untersuchungen der Atemmuskelfunktion an normalen Versuchspersonen. *Z ges exp Med* 1956 127: 89-92
15. J. M. SCHREIBER, J. M. SAMSON and C. SOULA: Etude électromyographique de la fatigue musculaire normale. *J Physiol (Paris)* 1954 46: 517-520
16. D. P. R. E. HYATT and D. L. FRY: An improved balloon system for measuring intraesophageal pressure. *J appl Physiol* 1959 14: 1057-1058
17. O. C. SMITH: Action potentials from single motor units in voluntary contraction. *Amer J Physiol* 1934 108: 629-638
18. M. R. SPIEGEL: Theory and problems of statistics. Schaum publishing company New York 1961
19. A. S. STEPANOV: Electromyogram changes produced by training in weight lifting. *Fiziol Zh (Mosh)* 1959 45: 129
20. TAYLOR A.: The contribution of the intercostal muscles to the effort of respiration in man. *J Physiol (Lond)* 1960 161: 390-402
21. T. TOKIZANE, T. J. KAWAMATA and H. TOKIZANE: Electromyographic studies on the human respiratory muscles. Studies on the activity pattern of neuromuscular units. *Jap J Physiol* 1952 2: 232-247
22. VILJANEN A.: The neural control of human respiratory muscles. *Acta physiol* and 1966 68 suppl 277: 212

- VILJANEN A A Electrical and mechanical activity of human respiratory muscles during voluntary inspiration Acta physiol scand 1967a in print
- The effect of CO<sub>2</sub> stimulation on the relationship between the electrical and mechanical activity in human intercostal muscles Scand J clin Lab Invest 1967 b in print
- H POPPIUS R M BERGSTRÖM and M HAKUMÄKI Electrical and mechanical activity in human respiratory muscles Acta neurol scand 1965 41 suppl 13 237—239
- WACHOLDER K und C MCKINLEY Über die Innervation und Tätigkeit der Atemmuskeln Pflügers Arch ges Physiol 1929 222 575—588
- WADE O L Movements of the thoracic cage and diaphragm in respiration J Physiol (Lond) 1954 124 193—212
- WEBER E Grundriss der biologischen Statistik VFB Gustav Fischer Verlag Jena 2 Auflage 1956
- WINTERSTEIN H Die automatische Tätigkeit der Atemzentren Pflügers Arch ges Physiol 1911 138 159—184
- WOLDRING S Interrelation between lung volume arterial CO<sub>2</sub> tension and respiratory activity J appl Physiol 1965 20 647—652
- WYSS O A M Impulssynchronisierung im Atmungszentrum Pflügers Arch ges Physiol 1939 241 524—538

## **APPENDIX**

TABLE I

NUMERICAL VALUES OF REGRESSION COEFFICIENTS AND CONSTANTS DERIVED FROM THE EQUATIONS  $n = b_n H_t + c_n$   
 $f_d = b_d W + c_d$  AND  $f = b P_t + c$  IN RESPECT OF THE EXPERIMENTAL SUBJECTS  $n$  = number of EMG impulses during  
 inspiration  $H_t$  = the first time integral of the inspiratory muscular work (cm H<sub>2</sub>O × sec × l)  $f_d$  = EMG impulse frequency at the end of  
 inspiration (sec<sup>-1</sup>)  $f$  = inspiratory muscular work (cm H<sub>2</sub>O × l)  $f$  = EMG impulse frequency of the static phase (sec<sup>-1</sup>)  $P_{st}()$  = alveolar  
 relaxation pressure (cm H<sub>2</sub>O)

Exper- mental subject	Number of pi- rations $N$	Regression coefficient $b$ cm H <sub>2</sub> O <sup>-1</sup> × l <sup>-1</sup>	Constant $a$	Regression coefficient $b_d$ cm H <sub>2</sub> O <sup>-1</sup> × sec <sup>-1</sup> × l <sup>-1</sup>	Constant $c_d$ sec <sup>-1</sup>	Regression coefficient $b$ cm H <sub>2</sub> O <sup>-1</sup> × sec <sup>-1</sup>	Constant $c$ sec <sup>-1</sup>	Corre- lation coeffi- cient $r$	Value of $t$	Stat sign $p <$	Modified standard error $ s_y $
1	10	6.4	57	0.2	2.9	13.4	-33.0	0.65	3.33	0.01	76.7
	10							0.86	6.04	0.001	18.4
	24							0.91	10.09	0.001	24.6
2	29	7.3	129.2	0.8	60.9	9.0	7.0	0.92	12.00	0.001	49.5
	20							0.91	9.31	0.001	16.3
	32							0.90	11.30	0.001	10.9
3	37	9.5	124.2	8.6	40.0	20.1	-14.5	0.80	7.63	0.001	116.2
	30							0.93	16.16	0.001	24.4
	34							0.92	17.21	0.001	2.3
4	12	9.3	-1.6	0.5	21.8	1.6	-71.2	0.93	8.00	0.001	49.1
	12							0.88	5.66	0.001	37.9
	25							0.90	9.90	0.001	20.3
5	16	4.2	71.2	3.9	62.0	8.8	7.7	0.93	9.47	0.001	27.4
	10							0.91	7.91	0.001	23.9
	17							0.89	7.36	0.001	23.9

6	16 20 10	86	413	39	485	108	-192	0.09 0.07 0.04	26.26 16.93 11.36	0.001 0.001 0.001	138 158 179
7	41 40 00	67	411	40	396	109	-245	0.04 0.00 0.00	17.1 13.08 7.59	0.001 0.001 0.001	254 279 220
8	19 17 00	60	43	40	71	105	-704	0.80 0.03 0.87	5.50 0.90 7.40	0.001 0.001 0.001	402 2.8 308
9	11 10 10	70	782	46	621	110	59	0.97 0.88 0.89	11.97 5.24 3.24	0.001 0.001 0.001	270 278 309
10	40 34 57	53	497	30	179	113	-712	0.83 0.86 0.06	0.17 0.53 25.43	0.001 0.001 0.001	463 203 145
11	16 12 12	86	40	30	203	115	-315	0.94 0.76 0.60	10.31 3.0 2.0	0.001 0.01 0.05	413 411 450
12	9 9 10	98	403	39	163	109	-17	0.93 0.96 0.96	13.03 9.07 9.70	0.001 0.001 0.001	252 208 162
13	35 37 35	61	211	44	-46	136	-1139	0.85 0.77 0.87	9.27 7.14 10.14	0.001 0.001 0.001	0.00 401 286
14	37 15 20	114	02	107	-400	197	-483	0.89 0.95 0.93	11.5 10.97 7.14	0.001 0.001 0.001	921 302 341



TABLE 2

THE EFFECT OF CO<sub>2</sub> STIMULATION ON THE ELECTRICAL AND MECHANICAL PARAMETERS IN THE INTERCOSTAL MUSCLES MENTIONED IN TABLE 1. A = before B = during and O = after (14 ± 2 min) CO<sub>2</sub> stimulation (5.0 ± 0.4% CO<sub>2</sub> air).  $t_b$  = value of  $t$  test by testing the significance of the difference of two regression coefficients  $t$  = similar value derived on testing significance of the two constants

Sub ject	Ex- peri- mental condi- tion	Num- ber of in- spi- ra- tions N	Regre- ssion coefficient $b$ $\text{cmH}_2\text{O}^{-1} \times$ $\text{sec}^{-1} \times 10^{-1}$	Con- stant $c$	Regre- ssion coefficient $b$ $\text{cmH}_2\text{O}^{-1} \times$ $\text{sec}^{-1} \times 10^{-1}$	Con- stant $c_d$ $\text{sec}^{-1}$	Pre- reson- coefficient $b$ $\text{cmH}_2\text{O}^{-1} \times$ $\text{sec}^{-1}$	Con- stant $c$ $\text{sec}^{-1}$	Value of $t_b$	Stat sign $p <$	Value of $t_e$	Stat sign $p <$	Corro- lation coeffi- cient $r$	Value of $t$	Stat sign $p <$	Modi- fied stan- dard error $ d_f $
1	A	8	9.6	41.9	0.0	-2.9	14.9	-67.8					0.00	8.40	0.001	33.7
		8											0.97	9.77	0.001	10.0
		9											0.89	4.78	0.01	31.3
	B	8	0.0	75.4	3.6	27.9	10.3	13.0	2.06		0.34		0.88	4.54	0.01	34.6
		8							3.96	0.01	1.66		0.99	17.16	0.001	12.6
		8							1.09		1.49		0.83	4.78	0.01	25.2
2	C	9	12.7	-11.4	5.8	56.7	10.1	-48.8	3.21	0.01	-1.4	0.00	0.14	7.23	0.001	33.0
		10							1.70		0.94		0.86	4.46	0.01	40.1
		10							1.16		1.79		0.90	6.64	0.001	23.0
	A	13	7.1	81.9	4.2	41.4	1.1	-74.6					0.86	3.33	0.001	44.4
		13											0.90	5.30	0.001	20.6
		10											0.81	3.91	0.01	31.2
3	B	14	5.3	106.8	3.5	70.2	13.7	-72.6	0.68		0.77		0.97	6.11	0.001	31.7
		14							0.64		1.12		0.79	4.46	0.001	30.2
		12							0.26		0.03		0.72	3.28	0.01	38.0
	C	14	0.3	6.1	3.2	59.9	13.7	-89.1	0.36		1.80		0.91	6.34	0.001	32.5
		14							0.35		0.40		0.80	5.30	0.001	25.2
		11							0.00		0.21		0.94	9.27	0.001	17.0

3	A	37 30 34	95	40.0	201	-187						0.80 0.95 0.0	7.89 16.10 17.21	0.001 0.001 0.001	1.07 21.4 22.3
	B	6 8 11	51	36.4	117	-341	7.0 6.3 0.17	0.01 0.001 0.001	0.46 0.84 0.00	0.01		0.87 0.79 0.95	4.3 3.0 0.13	0.01 0.0 0.001	0.07 18.1 17.1
	C	10 0 8	105	56.6	207	-31	3.0 4.14 0.43	0.01 0.001 0.001	1.06 0.00 0.11			0.32 0.08 0.09	6.64 13.03 17.19	0.001 0.001 0.001	0.3 15.3 9.7
4	A	1 1 3	93	21.8	106	-12						0.33 0.58 0.00	8.00 3.90 0.00	0.001 0.001 0.001	4.1 37.0 20.3
	B	0 9 8	70	13.0	157	-84.6	0.53 1.00 0.03		0.77 0.28 0.21			0.85 0.50 0.94	4.27 4.79 4.10	0.01 0.01 0.01	0.81 24.0 30.7
	C	10 10 7	117	-40.6	18.4	-103	1.6 5.09 0.66	0.001 0.001 0.001	0.30 2.02 0.29			0.71 0.00 0.97	0.1 9.70 8.9	0.001 0.001 0.001	0.80 20.3 10.2
5	A	16 1 17	4	60.0	99	77						0.93 0.01 0.01	9.47 7.91 7.50	0.001 0.001 0.001	7.4 3.0 23.0
	B	10 0 7	37	79.2	61	33	0.9 2.3 0.98	0.01 0.01 0.01	3.19 0.6 0.5	0.01		0.32 0.92 0.92	6.04 6.21 3.3	0.001 0.001 0.001	17.3 12.2 2.0
	C	12 1 11	88	64.2	107	-197	1.75 5.60 1.65	0.001 0.001 0.001	1.36 0.63 0.78	0.01		0.34 0.97 0.3	15.37 1.62 9.13	0.001 0.001 0.001	2.4 8.2 21.9
6	A	10 20 10	86	49.6	108	-19						0.09 0.97 0.97	9.6 16.93 16.45	0.001 0.001 0.001	13.8 1.8 17.9
	B	0 7 8	54	17.2	118	-9.6	1.49 0.39 0.40	0.0 0.0 0.0	0.0 1.00 0.16	0.0		0.85 0.93 0.10	4.27 5.00 3.08	0.01 0.01 0.01	60.8 36.7 20.6
	C	13 13 10	78	11.2	151	-137	1.40 0.09 0.9		2.04 0.14 0.91			0.82 0.82 0.89	5.3 4.7 5.52	0.001 0.001 0.001	63.9 47.0 34.2

Number of EMG impulses as a function of the pressure impulse produced by inspiratory muscles during one and the same inspiration expressed by equation  $n = b_1 I + c$  where  $b_1$  is the regression coefficient and  $c$  constant. The tables include the total number of EMG impulses ( $n$ ) duration of the inspiration ( $t$ ) total volume ( $V$ ) work of inspiratory muscles ( $W$ ) the first time integral of inspiratory muscular work ( $W_1$ ) the coefficient of linear correlation ( $r$ )  $A =$  before  $B =$  during and  $C$  after  $B \pm 0.4\%$   $CO_2$  stimulation. Each table (3 a b c d e f) relates to different subjects.

TABLE 3 a

Experimental condition	Inspiration	Number of points	Regression coefficient	Constant	Number of EMG impulses	Duration of inspiration (t, m)	Tidal volume	Work of inspiratory muscles	Physiological action in piratory muscles	Pressure impulse in piratory muscles	Correction coefficient
		$N$	$b$ $\text{cm H}_2\text{O}^{-1} \times \text{sec}^{-1}$	$c$	$n$	$t$ $\text{sec}$	$V$ $l$	$W$ $\text{cm H}_2\text{O} \cdot l$	$H_1$ $\text{cm H}_2\text{O} \times \text{sec} \times l$	$I$ $\text{cm H}_2\text{O} \times \text{sec}$	$r$
A	1	4	57	99	70	1.0	0.87	10.4	1	11.0	0.998
	2	5	70	70	100	1.31	0.77	7.9	5.5	13.3	1.000
	3	5	70	84	1.3	1.1	1.28	17.0	9.1	14.7	0.998
	4	6	67	14.5	261	1.35	2.86	80.8	5.0	36.4	1.000
	5	5	87	-0.1	251	1.17	2.32	62.3	34.7	29.2	1.000
	6	6	72	27	323	1.50	2.37	115.7	75.6	46.0	0.999
B	7	5	76	52	158	1.15	1.84	31.4	13.7	18.2	0.997
	9	6	75	159	208	1.36	2.12	43.2	23.3	26.5	1.000
	9	5	75	229	234	1.30	2.34	56.4	31.8	27.7	0.999
	10	5	77	187	205	1.15	2.45	55.2	28.8	24.5	0.999
	11	6	79	223	307	1.55	2.98	72.6	45.2	36.7	1.000
	12	8	68	292	472	2.00	4.06	139.0	113.3	65.0	0.999
C	13	5	71	102	76	1.10	0.72	6.4	5.0	9.2	0.999
	14	6	78	135	85	1.30	0.74	5.8	2.9	9.4	0.990
	15	5	86	102	137	1.00	1.12	13.1	7.0	14.6	0.999
	16	7	106	93	270	1.55	1.63	35.6	17.6	23.5	1.000
	17	6	112	104	301	1.60	2.06	37.2	27.7	29.9	1.000
	18	7	124	74	406	1.70	2.47	47.6	14.7	32.4	1.000

TABLE 36

Experimental condition	Inspiration points	Number of points	Regression coefficient $b$ $\text{cm H}_2\text{O}^{-1} \times 10^{-1}$	Constant $c$	Number of EMG impulses	Duration of inspiration $t$ sec	Tidal volume $V$ l	Work of inspiratory muscles $W$ $\text{cm H}_2\text{O} \times \text{l}$	Physical action of inspiratory muscles $H_i$ $\text{cm H}_2\text{O} \times \text{sec} \times \text{l}$	Pressure impulses produced by inspiratory muscles $I$ $\text{cm H}_2\text{O} \times \text{sec}$	Correction coefficient
A	1	8	3.4	3.3	45	1.80	0.89	0.3	4.5	11.7	0.997
	2	9	6.0	17.2	141	2.95	1.48	15.1	14.4	21.0	0.990
	3	9	8.6	9.4	918	2.30	1.3	19.2	17.7	23.7	0.999
	4	8	11.0	10.8	352	2.00	3.21	50.0	39.0	30.9	1.000
	5	8	7.3	10.9	198	2.00	2.28	3.7	2.3	25.2	0.987
	6	10	10.5	10	97	2.40	4.08	89.1	79.9	45.4	0.998
B	7	7	3.6	10.1	74	1.60	1.37	16.4	11.1	18.5	0.993
	8	8	5.2	12.7	108	1.80	0.33	49.8	45.7	30.4	0.999
	9	12	5.5	14.0	337	2.90	2.84	56.5	8.4	57.0	0.990
	10	7	5.4	16.6	135	1.65	1.34	30.4	20.6	23.0	0.986
	11	8	7.6	23.9	307	1.00	3.04	64.1	46.9	36.3	0.998
	12	10	10.3	17.5	6.3	2.45	3.98	105.2	94.0	55.0	0.995
C	13	6	7.0	7.8	6	1.50	0.77	9.0	3.5	10.1	0.990
	14	9	6	17.3	186	2.10	1.34	14.7	14.9	2.4	0.997
	15	10	11.1	9.2	400	2.50	2.24	34.2	35.2	34.1	0.998
	16	8	8.2	19.0	199	1.95	1.78	24.7	18.4	22.0	0.997
	17	7	8.8	16.7	937	1.75	2.10	34.4	23.7	24.7	0.997
	18	10	10.3	12.1	481	2.30	2.85	61.3	6.3	44.7	0.990

TABLE 3c

Experimental condition	Inspiration	Number of points	Regression coefficient	Constant	Numl or of EVG impulses	Duration of inspiration	Tidal volume	Work of inspiratory muscles	Physical action of inspiratory muscle	Pressure impulses produced by piratory muscles	Correlation coefficient
		<i>N</i>	<i>b</i> cm H <sub>2</sub> O $\Delta$ sec <sup>-1</sup>	<i>c</i>	%	<i>t</i> sec	<i>V</i> l	<i>W</i> cm H <sub>2</sub> O $\Delta$ l	<i>H<sub>i</sub></i> cm H <sub>2</sub> O $\Delta$ 100 g	<i>I</i> cm H <sub>2</sub> O	<i>r</i>
A	1	6	4.5	-12.6	73	1.60	13.5	1.5	12.4	15	0.907
	2	7	10.3	0.6	100	1.70	1.52	177	13.4	14.8	0.908
	3	8	11.3	7.2	30.2	2.00	1.6	210	24	25.9	0.939
	4	6	8.1	2.9	171	1.50	2.00	241	20.0	20	0.398
	5	8	10.6	-27.5	415	1.95	3.05	67.9	73.0	41	0.909
	6	10	11.2	1.6	604	2.45	3.37	55.0	118.1	59.9	0.939
B	7	7	4.9	12.8	103	1.70	1.06	13.4	10.4	19.2	0.352
	8	6	3.3	14.0	79	1.55	1.60	22.4	17.0	20.1	0.906
	9	9	7.9	22.1	271	2.10	1.85	29.4	31.0	30.8	0.908
	10	8	8.9	37.9	312	1.90	2.13	38.4	36.5	31.3	0.909
	11	8	9.0	0.1	353	1.00	2.90	67.9	57.1	38.0	0.903
	12	7	7.5	23.3	406	1.70	4.00	159.8	94.1	50.4	1.000
C	13	6	4.8	8	72	1.45	1.00	9.7	7.2	13.9	0.906
	14	7	4.8	10.6	87	1.60	1.02	10.4	8.1	15.1	0.906
	15	7	8.5	4.5	185	1.60	1.50	21	17	20.8	0.939
	16	7	12.3	12.4	308	1.70	1.50	22.3	19.5	23.4	1.000
	17	6	12.1	-3	295	1.55	1.75	29.1	24.4	24.1	0.909
	18	11	11.4	-29.1	555	2.60	2.45	48.3	75.5	49.2	0.906

TABLE 3d

Experimental condition	Inspiration points	Reg. s. m. coefficient	Constant	Number of FMG impulses	Duration of inspiration, sec	Total volume, l	Work of inspiratory muscles, cm H <sub>2</sub> O $\times$ l	Physiologic action of inspiratory muscles, cm H <sub>2</sub> O $\times$ sec $\times$ l	Pressure impulse produced by inspiratory muscles, cm H <sub>2</sub> O $\times$ sec	Correlation coefficient
	<i>N</i>	<i>b cm H<sub>2</sub>O <math>\times</math> 10<sup>-2</sup></i>	<i>c<sub>i</sub></i>	<i>n</i>	<i>t</i> sec	<i>V</i> l	<i>W</i> cm H <sub>2</sub> O $\times$ l	<i>H<sub>i</sub></i> cm H <sub>2</sub> O $\times$ sec $\times$ l	<i>I</i> cm H <sub>2</sub> O $\times$ sec	<i>r</i>
A	1	4	11.2	20.9	89	1.10	0.87	2.7	0.2	0.997
	2	5	12.1	18.4	158	1.40	1.03	7.7	11.5	1.000
	3	6	15.1	0.6	109	1.60	1.20	8.0	12.4	0.998
	4	0	17.5	18.4	4.0	2.30	1.30	20.4	22.4	0.999
	5	7	21.4	15.8	481	1.90	1.74	20.4	20.0	0.999
	6	8	18.1	11.4	484	2.10	2.03	33.8	20.1	1.000
B	7	4	10.3	8.4	104	1.10	1.04	6.0	9.3	1.000
	8	5	10.8	13.7	114	2.10	1.00	9.2	16.4	1.000
	9	8	9.4	0.0	161	2.10	1.25	9.8	16.3	1.000
	10	6	9.9	5.3	169	1.40	2.24	18.1	17.1	0.999
	11	9	10.5	18.8	307	2.30	2.02	34.4	27.5	0.999
	12	13	10.2	16.2	37	3.00	2.28	21.2	40.7	0.999
C	13	6	10.4	-3.7	172	1.40	1.08	3.9	8.1	0.998
	14	5	10.0	10.0	109	1.00	1.28	4.0	8.3	0.997
	15	9	14.6	15.6	203	2.30	1.49	13.3	19.1	0.999
	16	7	19.6	19.3	37	1.00	1.08	10.0	13.0	0.998
	17	7	17.1	13.7	301	1.00	2.12	17.0	16.7	0.999
	18	8	21.4	18.1	471	3.00	2.84	25.4	21.4	1.000

TABLE 3e

Experimental condition	Inspiration	Number of points	Regression coefficient $b$ $\text{cm H}_2\text{O}^{-1} \times 100^{-1}$	Constant $c_1$	Number of EMG unspikes	Duration of inspiration $t$ sec	Total volume $V$ $l$	Work of inspiratory muscles $W$ $\text{cm H}_2\text{O} \times l$	Physical action of respiratory muscles $H_c$ $\text{cm H}_2\text{O} \times \frac{1}{\text{sec} \times l}$	Measure unspikes produced by inspiratory muscles $I$ $\text{cm H}_2\text{O} \times \frac{1}{\text{sec}}$	Correlation coefficient $r$
A	1	6	3.5	4.0	42	1.50	0.58	3.9	3.1	11.1	0.998
	2	8	4.2	12.5	86	2.03	0.88	8.0	7.7	18.4	0.991
	3	7	5.6	10.2	121	1.81	1.14	12.4	11.2	20.2	0.996
	4	9	7.1	21.2	199	2.19	1.34	15.8	16.0	20.7	0.997
	5	8	8.4	16.8	260	2.10	1.99	29.8	26.5	29.6	0.999
	6	13	8.9	-6.4	737	3.16	4.43	116.2	152.3	9.9	0.998
B	7	5	9.0	-9.3	163	1.28	1.14	20.0	13.4	27.7	0.999
	8	5	6.1	0.1	160	1.30	1.31	28.8	21.1	27.6	0.999
	9	6	8.5	-1.5	345	1.50	1.78	50.4	37.3	40.0	1.000
	10	7	8.8	18.7	483	1.65	1.94	65.6	46.4	51.7	0.999
	11	7	8.4	9.6	488	1.70	1.96	65.1	70.0	54.5	0.997
	12	8	7.6	-0.2	515	2.00	2.69	94.6	93.1	67.0	1.000
C	13	9	9.0	18.2	120	2.30	0.91	8.0	10.2	22.4	0.993
	14	6	9.7	18.1	120	1.50	1.06	13.0	10.0	17.7	0.993
	15	9	7.2	22.4	295	2.31	1.92	32.2	35.9	36.3	0.997
	16	7	9.5	25.7	344	1.82	2.04	39.1	36.2	33.2	0.998
	17	9	6.8	16.8	277	2.20	2.00	37.0	43.0	37.6	0.999
	18	8	8.4	11.0	468	2.04	3.20	96.6	90.5	52.8	0.999

TABLE 3f

Experimental condition	Number of points	Regression coefficient	Constant	Number of FPG impulses	Duration of inspiration	Total volume	Work of inspiratory muscles	Physiologic action of inspiratory muscles	Pressure of inspiration by inspiratory muscles	Correction coefficient
	<i>N</i>	$b$ $\text{cm H}_2\text{O}^{-1} \times \text{sec}^{-1}$	<i>c</i>	<i>n</i>	<i>t</i> sec	<i>V</i> l	$W$ $\text{cm H}_2\text{O} \times \text{l}$	$H_i$ $\text{cm H}_2\text{O} \times \text{sec} \times \text{l}$	$P$ $\text{cm H}_2\text{O} \times \text{sec}$	<i>r</i>
A	1	11.0	2.6	1.0	1.50	1.16	10.2	6.8	11.0	0.995
	2	14.7	2.1	2.5	1.00	1.90	21.3	12.0	15.0	0.994
	3	13.0	9.0	3.3	1.60	2.00	37.9	31.9	23.8	0.999
	4	13.1	35.0	5.84	2.25	3.85	76.6	88	41.0	0.990
	5	11	37.8	7.03	2.40	4.75	120.5	141.9	58.8	1.000
	6	12.1	3.5	1.74	1.30	1.19	21.2	10.8	14.0	1.000
B	7	10.7	2.6	1.80	2.00	2.07	32.9	30.4	31.0	0.994
	8	10.4	2.2	3.99	1.70	2.18	64.8	53.2	34.0	0.999
	9	10.8	32.1	4.33	1.90	3.30	72.4	9.8	37.2	0.991
	10	7.7	24.9	5.14	2.00	4.90	166.0	131.1	64.0	1.000
	11	7.0	17.6	10.7	1.40	1.04	10.3	7.5	12.8	0.993
	12	10.8	37.3	4.02	1.60	2.08	0.0	0.2	93.0	0.991
C	13	11.0	37.7	21.0	1.00	1.70	16.3	1.2	14.3	0.990
	14	13.0	30.8	30.2	1.00	2.61	96.0	4.1	20.2	0.998
	15	10.3	19.7	16	1.0	1.4	11.5	10.3	13.7	0.995
	16	18.1	30.3	4.0	1.60	2.8	9.2	1.2	20.3	0.996
	17	13.0	30.8	4.0	1.30	1.1	1.1	1.1	1	1.000
	18	10.9	18.4	2.7	2.00	1.1	1.1	1.1	4	0.999









**POSTNATAL CHANGES  
IN CUTANEOUS REFLEXES  
AND IN THE DISCHARGE PATTERN  
OF CUTANEOUS AND ARTICULAR  
SENSE ORGANS**

**A MORPHOLOGICAL AND PHYSIOLOGICAL STUDY  
IN THE CAT**

**BY**

**JAN EKHOLM**



ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 297

FROM THE DEPARTMENT OF ANATOMY KAROLINSKA INSTITUTET STOCKHOLM 60 AND  
THE DEPARTMENT OF HUMAN ANATOMY UNIVERSITY OF UPPSALA SWEDEN

POSTNATAL CHANGES  
IN CUTANEOUS REFLEXES  
AND IN THE DISCHARGE PATTERN  
OF CUTANEOUS AND ARTICULAR  
SENSE ORGANS

A MORPHOLOGICAL AND PHYSIOLOGICAL STUDY  
IN THE CAT

BY

JAN EKHOLM

UPPSALA 1967



# CONTENTS

CHAPTER I <i>General Introduction</i>	7
CHAPTER II <i>Anatomy and nomenclature</i>	9
CHAPTER III <i>Segmental origin of cutaneous nerves</i>	13
Introduction and literature	13
Material and Methods	14
Results	14
Discussion	17
Summary	17
CHAPTER IV <i>On the distribution of hindlimb dermatomes</i>	19
Introduction	19
Material and Methods	19
Results	22
Discussion	22
Summary	23
CHAPTER V <i>Calibre spectra of cutaneous and articular nerves during postnatal development</i>	24
Introduction	24
Material and Methods	24
Results	26
A Adult cats	26
B Kittens	27
C Total number of myelinated fibres	35
Discussion	35
Summary	38
CHAPTER VI <i>Conduction velocity of cutaneous nerves during postnatal development</i>	39
Introduction and literature	39
Material and Methods	40
Results	40
Discussion	44
Summary	46



<i>organs during postnatal development</i>	48
Introduction and literature	48
Material and Methods	50
Results	51
A Recording from the sural nerve	51
II Hair movement sensitive afferent units	52
1 Earlier observations on adult units	52
2 Present observations	53
Mean frequency of impulse train	54
Electrical stimulation	55
Repeated stimulation	55
Receptive fields	56
C Thermosensitivity of afferent units	60
1 Earlier observations	60
2 Present observations	61
D Touch units in hairy skin	62
1 Earlier observations on adult units	62
2 Present observations	62
Reproducibility of response	68
Strong and weak stimulation	70
Number of spikes	71
Electrical stimulation in the skin	71
E Articular units	73
Single units in dorsal roots	73
Recording from medial knee joint nerve	74
Strong and weak stimulation	75
Discussion	77
Summary	80
 <b>CHAPTER VIII <i>Cutaneous reflexes during postnatal development</i></b>	 85
Introduction and literature	83
Material and Methods	85
I Experiments with electrical stimulation of n. suralis	85
II Experiments with mechanical stimulation of the skin	87
A Monosynaptic test reflexes	87
II EMG recordings	89
Results	89
A The effect of single shock stimulation of a cutaneous nerve on ipsilateral monosynaptic test reflexes	89
1 Early observations on adult cats	89
2 Present observations	90
II The effect of iterative stimulation of a cutaneous nerve on monosynaptic test reflexes in kittens	92
1 Earlier observations on adult cats	92
2 Present observations	92

C	<i>The effect of mechanical stimulation of different skin areas on monosynaptic test reflexes</i>	94
	Comments	97
D	Observations of reactions to skin stimulation in kittens	98
E	Excitatory and inhibitory skin areas for extensor and flexor muscles as studied with EMG	99
1	Postnatal changes in the general response in the muscles	99
2	Ankle extensors	99
3	Ankle flexors	103
4	Spinalization	107
5	Knee extensors and flexors	107
6	Responses to stimulation of the contralateral heel	108
	Discussion	108
	Summary	113
CHAPTER IX	<i>General discussion</i>	116
	General summary	120
	Acknowledgments	123
	References	124

CHAPTER VII <i>Discharge patterns of cutaneous and articular sense organs during postnatal development</i>	48
Introduction and literature	48
Material and Methods	50
Results	51
A Recording from the sural nerve	51
II Hair movement sensitive afferent units	52
1 Earlier observations on adult units	52
2 Present observations	53
Mean frequency of impulse train	54
Electrical stimulation	55
Repeated stimulation	55
Receptive fields	56
C Thermosensitivity of afferent units	60
1 Earlier observations	60
2 Present observations	61
D Touch units in hairy skin	62
1 Earlier observations on adult units	62
2 Present observations	62
Reproducibility of response	68
Strong and weak stimulation	70
Number of spikes	71
Electrical stimulation in the skin	71
E Articular units	73
Single units in dorsal roots	73
Recording from medial knee joint nerve	74
Strong and weak stimulation	75
Discussion	77
Summary	80
CHAPTER VIII <i>Cutaneous reflexes during postnatal development</i>	85
Introduction and literature	83
Material and Methods	85
I Experiments with electrical stimulation of n. suralis	85
II Experiments with mechanical stimulation of the skin	87
A Monosynaptic test reflexes	87
II EMG recordings	89
Results	89
A The effect of single shock stimulation of a cutaneous nerve on ipsilateral monosynaptic test reflexes	89
1 Early observations on adult cats	89
2 Present observations	90
II The effect of iterative stimulation of a cutaneous nerve on monosynaptic test reflexes in kittens	92
1 Earlier observations on adult cats	92
2 Present observations	92

## CHAPTER I

# GENERAL INTRODUCTION

Following the neuro histological studies of the embryonic central nervous system by *His* (1889) and *Ramon y Cajal* (1890 a b) which led to the formulation of the neurone theory the physiology of developmental mechanisms attracted great interest. During the first four decades of this century several extensive investigations on this subject were published some concerning behavioral aspects (e.g. *Graham Brown* 1914, 1915 *Coghill* 1914 1916 1926 a b 1930, *Weed* 1917, *Langworthy* 1925, 1926 1929, *Minkowski* 1921 a, b 1923 *Windle* 1929 1934 1940, *Angulo y González* 1932 1937 *Coronios* 1933 *Carmichael* 1933 1934 a b *Hooker* 1937). Since the contemporary physiological techniques were rather crude both in comparison with those now available and compared with the morphological techniques of that time no detailed analyses of the mechanisms behind developmental changes were possible.

The physiological studies consisted chiefly of determinations of when and where in the organism different reflexes appeared or disappeared during the process of development. These findings are obviously indispensable for studies of developmental mechanisms but give no information about the developmental changes in different parts of a reflex. With modern electrophysiological techniques however, it is possible to study separately both the afferent and the efferent links of a reflex in detail and thereby uncover the links in a chain of developmental events.

In 1960 *Skoglund* published a series of important papers regarding the postnatal development of postural reflexes (for review see *Skoglund* 1966). He found that the decerebrate rigidity and tonic stretch reflexes are absent in the hindlimbs of newborn kittens, whereas motor activity is easily elicited from the skin demonstrating that the efferent side of the stretch reflex path is functioning. He further showed that the muscle sense organs only give phasic discharges of low frequency at birth and that tonic discharges do not appear until the conduction velocity of the afferent fibres has reached a certain level. These findings explain the absence of tonic stretch reflexes in newborn kittens especially since it was also found that the muscle spindles lack gamma innervation for some time after birth which provides the explanation for the absence of decerebrate rigidity (*Granit* 1955). The

effectiveness of skin stimuli as against activation of muscle receptors in evoking motor responses in the newborn kitten poses several questions, two of which constitute the main theme of this thesis

1) Are the cutaneous sense organs functionally more mature than the muscle receptors which would explain their greater influence on motor activity?

2) Are the cutaneous sense organs as immature functionally as the muscle receptors and do their properties change with the same relationship between function and fibre size as in muscle sense organs? If so is their greater effectiveness in evoking motor activity a consequence of the absence or immaturity of supraspinal regulation and segmental postural reflexes?

In order to throw light on these questions a study has been made of functional changes of the cutaneous sense organs the morphological development of such nerve fibres and alterations in the patterns of spinal reflexes elicited from the skin in cats of different stages of maturation from newborn to adult

## ANATOMY AND NOMENCLATURE

For the convenience of the reader a description of the gross anatomy of the relevant cutaneous nerves and the nomenclature used is presented. Most of the information is available in the literature (Stowell 1891 Horsburgh and Heath 1950 Reighard and Jennings 1961 Harrison 1962) but some new observations have been added. Most of these were made in connection with the preparation of the animals for experiments or biopsy but some animals were also dissected systematically. When the innervation fields of the skin nerves were determined the nervous activity set up by mechanical stimulation of the skin was recorded from the proximal parts of the nerves (for method of recording see Chapter III). The mechanical stimulation was performed by means of a sable brush (Chapter IV).

The nomenclature used has been adapted from the textbooks mentioned above and generally modified according to the *Nomina Anatomica* (1964).

The following cutaneous nerves have been of interest in the present study (the abbreviations used are given in brackets).

*N cutaneus femoris lat* (n cut fem lat) whose branches are distributed over the lateral aspect of the thigh extending distally to the knee joint region enters the thigh by penetrating the abdominal wall near spina iliaca ant sup (Fig 1). It accompanies a ilio lumbalis and is often divided into two main trunks.

*N saphenus* is a branch of n femoralis. It passes initially along a femoralis (Fig 1) and more distally on the medial side of the knee joint and along the lower limb. Its end branches extend to the ankle joint region and to the dorsum of the foot.

*N cutaneus femoris post* (n cut fem post) is usually found between tuber ischi and trochanter major (Fig 1) where the nerve emerges through the muscles. It gives off branches in medial and lateral directions. The main branch continues along the popliteal fossa. It usually extends distally on the posterior part of the calf accompanying v saphena parva (cf Reighard and Jennings 1961).

*N suralis* usually originates from n tibialis or from n ischiadicus (sciatic nerve) proximal to its division into n tibialis and n peroneus communis. *N suralis* pierces the adipose tissue in the popliteal fossa and lies usually on

the posterior surface of m gastrocnemius (Fig 1) The main branch passes to the heel where it gives off twigs for the heel region and the latero plantar aspect of the foot Some twigs are often crossed by twigs of n cut fem post, the latter pass medially more superficially than the branches of n suralis A lateral branch sometimes originating from n suralis and sometimes directly from n tibialis or n ischiadicus often gives off branches in the lateral part of the knee joint region and the lower limb

*Nn plantaris med et lat* are branches of n tibialis and emerge from behind the medial malleolus The nerves give off branches to the plantar surface of the foot and toes

*N peroneus superficialis* (n per superf) becomes superficial near the ankle joint region on the anterior side of the lower limb It supplies the ankle region the dorsum of the foot and the toes

*N peroneus profundus* (n per prof) gives off some cutaneous branches on the dorsum of the tarsus and supplies the lateral digits

*Variations observed in the present study*

1) Anastomoses were often noticed between n suralis and n cut fem post between n suralis and n per superf between n cut fem post and n per superf and between n per superf and n saphenus It appeared as if fibres of the distal branches of the different nerves came together and then ran in a common strand

2) In three out of some 150 preparations n suralis was completely embedded in muscular tissue for about 1 cm of its length in the proximal part of m gastrocnemius In none of these cases was the nerve embedded in the gastrocnemius muscle on the opposite side

3) N cut fem post varied in its length According to *Reighard and Jennings* (1961) it may be traced as far distal as the popliteal space In the present study however it was found that in most cases this nerve extended down to and sometimes beyond the heel region There seemed to be a relationship between the magnitude of n suralis and n cut fem post When n suralis was thin and extended only a short distance distally n cut fem post was large and gave off branches far distally in the area that n suralis normally supplied In 2 cases out of some 150 the main branch (medial) of n suralis could not be found but n cut fem post was large and supplied the area The opposite situation was also seen when n cut fem post was small and n suralis was large In such variations were unilateral the other side having the opposite pattern of distribution

*Innervation fields of the cutaneous nerves*

The skin area supplied by the cutaneous nerves was established exactly by dissection The innervation fields of the cutaneous nerves in the lower limb

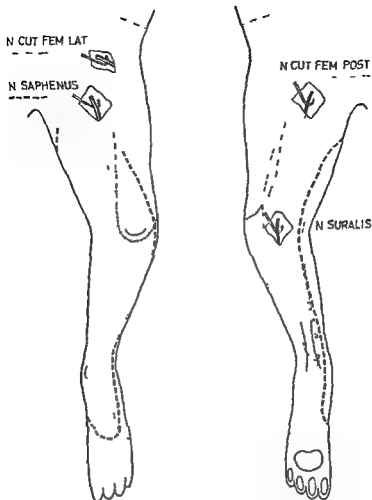


FIG 1 Diagram showing the innervation fields and proximal main trunks of cutaneous nerves on the anterior and posterior aspects of the hindlimb

cutaneous nerves in the cat was performed by means of an electrophysiological technique

Fig 1 shows the innervation fields of different cutaneous nerves on the anterior and posterior aspects of the hindlimb in a cat. *N. saphenus* supplied a large area on the antero-medial aspect of the thigh, lower limb and proximal part of the dorsum of the foot. In one case this nerve consisted of two naturally separated fasciculi. These two fasciculi were investigated separately and it was found that there was very little overlapping between the two innervation fields. One of the natural fasciculi



was then artificially divided into two new fasciculi of approximately equal thickness. The innervation fields of the two new artificial fasciculi were tested separately and it was found that these two fields were almost identical. *N cut fem lat* supplied an area on the lateral aspect of the thigh. This area extended far enough anteriorly and posteriorly to overlap somewhat on the areas of the *n saphenus* and *n cut fem post*. On the posterior surface of the hindlimb the area supplied by *n cut fem post* can be seen. This area is situated on the posterior surface of the thigh and lower limb. *N suralis* usually supplied the postero lateral part of the lower limb, the heel region and the lateral margin of the foot. The distal border of the field varied, sometimes it extended to the middle of the foot, sometimes to the lateral toe. Some overlap between different areas subserved by the nerve trunks was observed but this was not as great as that of the dermatomes (see Chapter IV). The innervation fields of the cutaneous nerves studied were not exactly bisymmetrical.

The following terms will be used

buttock	= the caudal region ventro lateral to the base of the tail
thigh	= the part of the hindlimb between the hip and knee joints
lower limb (leg)	= the part of the hindlimb between the knee and ankle joints
heel	= the region at the posterior end of the calcaneum
foot pad	= plantar cushion = the central pad of the sole of the foot
toe pads	= the peripheral pads on the toes
rootlet	= naturally occurring subdivisions of spinal roots at the entrance into the spinal cord
afferent unit	= peripheral afferent unit (sensory unit) = afferent axon, its receptor terminals or endings and any associated non nervous structural elements (Iggo 1966 p 240)

## SEGMENTAL ORIGIN OF CUTANEOUS NERVES

*Introduction and literature*

*Stowell* (1882 1886, 1887 1891) dissected the peripheral nervous system in the cat. In 1891 he described the lumbo sacral plexus and its peripheral nerves. With his macro dissection method it was only possible to study the segmental origin of such nerves that originated from the plexus separately. The segmental origin of branches to larger nerves (e.g. *n. suralis* and *saphenus*) could not be traced by this method. The segmental origin of *n. cut. fem. lat.* was reported to be L5—L6. Elsewhere in the text, however *Stowell* reported: 'The exposure of the myelin in the arch of the caudal thoracic vertebra (thirteenth) exposes the ectal origin of the first lumbar nerve. This probably means that the nerve that he denominated L1 is the same one as is usually denominated Th 13. An analogous situation seems to exist with *Stowell's* description of the first sacral nerve: the largest of the spinal nerves, *n. cut. fem. post.*, it traverses the long groove in the seventh lumbar vertebra and finds its exit through the foramen mesad of the crista illi. The root described in this way must be the one usually denominated L7.'

According to *Stowell* (after correction of the root number) *n. cut. fem. lat.* should originate from L4—L5. This is consistent with some textbooks of cat anatomy (*Reighard and Jennings* 1961, *Harrison* 1962).

*N. cut. fem. post.* has been reported to originate from S2—S3 (*Reighard and Jennings* 1961) and from S1—S3 (*Harrison* 1962).

The segmental origin of cutaneous nerves has been investigated also by means of electrophysiological techniques. *Widen* (1951 (cited by *Hagbarth* 1952)) showed that *n. suralis* and *n. cut. fem. post.* could be mainly referred to dorsal roots S1 and L7 while *n. saphenus* could be mainly referred to L6 and L5.

In the present work recordings had to be made from dorsal root fibres connected to cutaneous sense organs (Chapter VII). It then had to be determined where the cutaneous afferents of a certain nerve or from a certain region (Chapter II) reached the spinal cord. Furthermore, for the study of cutaneous reflexes (Chapter VIII) knowledge of the segmental

origin of the stimulated nerves was of value. Since the results presented in the literature concerning these questions are incomplete and somewhat controversial, the present study was performed with the two following questions in mind: 1) Into what dorsal roots do fibres from a certain cutaneous nerve enter? 2) Are the subdivisions of the dorsal root i.e. the dorsal rootlets organized in such a way that fibres of a certain nerve can be constantly found in one or more certain rootlets?

## *Material and Methods*

Seven adult cats were used and denominated by consecutive numbers. They were anaesthetized with pentobarbital 40 mg/kg body weight (b.w.) intraperitoneally (i.p.). A lumbo-sacral laminectomy was performed. The dura was divided and the dorsal roots L3—S3 were cut close to the spinal cord and immersed in liquid paraffin. In the hindlimb the cutaneous nerves were dissected free and covered with liquid paraffin. The animals were mounted on a conventional experiment stand. The rectal temperature was checked and maintained near to 37°C by means of adjustable radiant heat. In cats Nos. 1, 2 and 3 the rootlets were placed on Ag—AgCl hooks and stimulated and the responses were recorded from the peripheral nerves. The recording electrode (Ag wire) on the peripheral nerve was always placed proximal to the branches of the nerve. A reference electrode was inserted into inactive tissue. In cats Nos. 4, 5 and 6 the peripheral nerves were stimulated and the recording was made from the rootlets. The two methods gave essentially similar results.

The stimulation was performed by square wave pulses of 0.3 msec duration. The stimulus strength was supramaximal. The potentials were recorded between the active electrode on which the nerve was placed and the reference electrode inserted into inactive tissue through a cathode follower input to an amplifier, displayed on a cathode ray tube and photographed on moving bromide paper. Time was indicated by a sine wave generator on a second beam.

## *Results*

The segmental origins of the suralis, cut fem post, saphenus and cut fem lat are schematically illustrated in Figs. 2—3. Naturally occurring rootlets that were found to have connections with the cutaneous nerve studied are indicated by the black areas in the graphs. The length of each

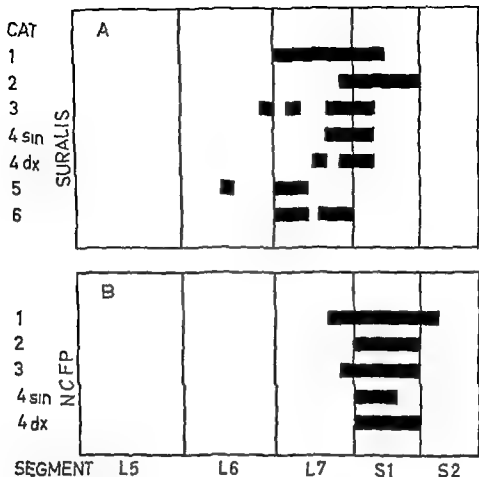


FIG. 2 Diagram showing the segmental origin of n. suralis (A) and n. cut fem post (B). The black areas indicate rootlets found to have connections with the cutaneous nerve.

segment in the graphs is roughly proportional to the root entrance zone (Thomas and Combs 1962).

The segmental origin of n. suralis in 6 cats is shown in Fig. 2A. In one cat both sides were studied (No. 4). It can be seen that the fibres of the sural nerves studied originated from the L6, L7 and S1 dorsal roots. In each individual animal the origin usually comprised only two segments: L7 and S1 in four cases and L6 and L7 in one case. Such variations are probably due to the type of pre- and postfixation of the cats. In one cat however L6, L7 and S1 were involved and in one case only L7. It can

also be seen in Fig. 2A that some fibres of n suralis originated from L7 in all cases. Fibres of n suralis did not originate from consecutive rootlets in cats No 3, 4, 5 and 6. In cat No 2, for instance there were three rootlets between the two portions of the segmental origin. In cat No 3 the origin was divided into three portions. In cat No 1 the number of rootlets of each dorsal root studied was less than usual, each rootlet was however thicker than usual. In this case no interruption of the origin was observed. These findings probably depend not only upon the distribution of the fibres but also upon the thickness of the naturally occurring rootlets from which the recordings were made.

In the most caudal rootlets of L7 fibres of n suralis were found in 6 cases out of 7. This finding that fibres can nearly always be found in the most caudal part of L7 (and rostral part of S1) was utilized and confirmed in the work presented in chapter VII. The results obtained in cat No 4 showed that the segmental origin of n suralis was not equal on the two sides, which may be a common situation.

Fig. 2B shows that n cut fem post originated from L7, S1 and S2. In 3 cases out of 5 only S1 was involved. Fibres of this nerve were found in

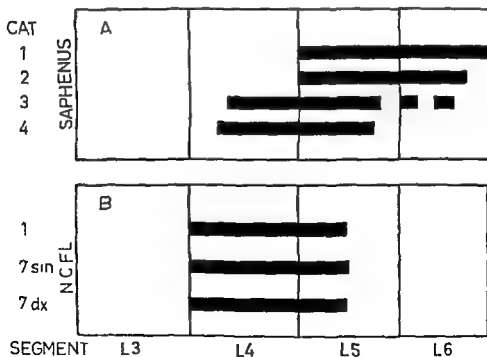


FIG. 3 Same as Fig. 2. N. suralis (A) and n. cut fem lat (B).

the rostral part of S1 in all cases studied. The origin of the nerve in cat No 4 was not bisymmetrical.

Fibres of *n. saphenus* (Fig. 3A) were traced to the dorsal roots L4, L5 and L6. In two cases L5 and L6 were involved, in one L4 and L5 and in one L4, L5 and L6. Saphenous fibres were found in the rostral part of L5 in all cases studied.

Fig. 3B shows the results obtained in the experiments on *n. cut. fem. lat.* In all three cases the fibres of this nerve were found in L4 and the rostral part of L5 (in one case L3 was not tested).

## Discussion

When using a method such as this for tracing nerve fibres, one has to consider the possible errors involved. The ratio between active and inactive fibres at the recording electrode is of importance. If the ratio is small, the activity of a few fibres might be completely shunted. By using recording and stimulation at both ends of the fibres, an attempt has been made to minimize such errors.

The segmental origins of *n. cut. fem. lat.*, *n. suralis* and *n. saphenus* found in the present study are essentially consistent with the reports in the literature.

The segmental origin of *n. cut. fem. post.* however was not quite in accordance with those reported in the anatomical textbooks (S2—S3: *Reighard and Jennings* 1961 and S1—S3: *Harrison* 1962). This nerve was found to originate mainly from S1 and the caudal part of L7 and only in one case from the rostral part of S2. Fibres of this nerve were never found in the dorsal root S3. The findings are essentially in accordance with those of *Widen* (cited by *Hagbarth* 1952). Thus, in contrast to other reports, *Widen's* finding was confirmed. However, some features of the segmental origin of cutaneous nerve fibres ought to be stressed.

1. The composition of the segmental origin varies considerably between the specimens. 2. Fibres of one cutaneous nerve do not always enter consecutive rootlets. 3. On the other hand, some fibres of a particular nerve often enter a certain part of a root or a certain rootlet.

## Summary

1) The segmental origin of different hindlimb cutaneous nerves was studied by electrical stimulation of nerves or rootlets and recording from rootlets or nerves in cats.

2) N suralis originated from L6 L7 and S1 but in each animal usually only from two of these segments. Fibres of the sural nerve did not enter consecutive rootlets. However sural fibres were nearly always found in the caudal rootlet of L7 (and rostral S1).

3) N cut fem post originated from L7 S1 and S2 but usually from S1. Fibres of this nerve were always found in the rostral part of S1.

4) N saphenus originated from L4 L5 and L6 saphenous fibres were always found in the rostral part of L5.

5) N cut fem lat originated from L4 and L5.

## ON THE DISTRIBUTION OF HINDLIMB DERMATOMES

### *Introduction*

The distribution of the dermatomes in the cat was investigated by *Klessens* (1914). He used a strychnine isolation technique: hyperreflexive zones (which corresponded to the dermatomes) were mapped out by touching the skin after the application of strychnine to the dorsal root under study. The adjacent dorsal roots were sectioned. *Kuhn* (1953) and *Hekmatpanah* (1961) used an electrophysiological technique for defining the tactile dermatomes. They initiated afferent impulses by mechanical stimulation of the skin and recorded the responses from the sectioned dorsal rootlets. The muscle nerves were not sectioned. *Kuhn* investigated the segments from L4 to S4 in the cat and *Hekmatpanah* investigated the segments from C1 to L4, also in the cat. By recordings from dorsal root afferents *Pubols et al.* (1965) made observations of the forelimb dermatomes in the cat.

The results of *Klessens* (1914) and *Kuhn* (1953) are not quite in accordance. *Kuhn* found that the S1 dermatome innervated only the buttocks and the base of the tail, whereas *Klessens* found that it extended more distally. The observations of *Kuhn* are those usually cited in the literature (*Celesia* 1963).

When studying the cutaneous reflexes (Chapter VIII) skin stimuli were applied to different regions of the hindlimb and it was therefore important to know which segment supplied the region under study; hence the distribution of some hindlimb dermatomes was reinvestigated here.

### *Material and Methods*

Cats and kittens were anaesthetized with pentobarbital 40 mg/kg b.w. Since the material was obtained from the same animals that were used for the investigation of the cutaneous sense organs (Chapter VII) it comprised about 100 cats and kittens. A lumbo-sacral laminectomy was performed and the dorsal roots L6—S1 were exposed and sectioned near the spinal cord (for details see Chapter III). Nn. tibialis and peroneus communis



were cut in order to avoid interference from muscle sense organs. This meant that the cutaneous receptive areas of the nn plantares and nn per superfi and prof were not included in the dermatomes mapped. The configuration presented of the distal ends of the dermatomes on the foot is thus not anatomically correct: the true dermatomes may be somewhat larger. The proximal parts of the dermatomes near the trunk were not completely investigated.

Nerve fibres that were connected to cutaneous sense organs were isolated in dorsal roots by dissecting free thin filaments under the microscope. Recording from single dorsal root filaments is described in more detail in Chapter VII. The recording equipment was the same as described in Chapter III. Stimulation of the skin was usually performed by means of a sable brush and the localization of the receptive fields of the single mechanosensitive units was determined. The localization was indicated on drawings.

In 4 cats the size and localization of the S1 dermatomes were determined by recordings from the rootlets. The muscle nerves were cut (see above) and the borders of the dermatomes were mapped by means of mechanical stimulation of the skin with a sable brush.

## Results

Figs 4A and 5A C show the skin areas within which receptive fields of mechanosensitive units of the dorsal roots L6, L7 and S1 were localized. The observations were made in several different cats and the areas attributed to the different segments represent a sum of these observations. The variation between the specimens is thus included. As can be seen in Fig 4A the S1 dermatome was situated on the posterior aspects of the thigh, calf and foot and extended to the distal part of the foot. Sometimes units were also found down to the digits. Fig 5A shows the L7 dermatome. It had a similar localization but was shifted somewhat laterally as compared with S1. The L6 dermatome (Fig 5B C) was incompletely mapped but it can be seen that part of the dermatome covered the anterior and medial aspects of the lower leg and that this dermatome also extended to the digits.

Fig 4B shows the S1 dermatome in one of the cats in which it was mapped by recording from dorsal rootlets. The dermatome extended distally to the middle of the plantar surface of the foot. Except for the foot the area is essentially in congruence with that obtained by the single unit method (Fig 4A). The dermatomes obtained by recordings from rootlets in the rest of the cats were essentially similar to that illustrated in Fig 4B.

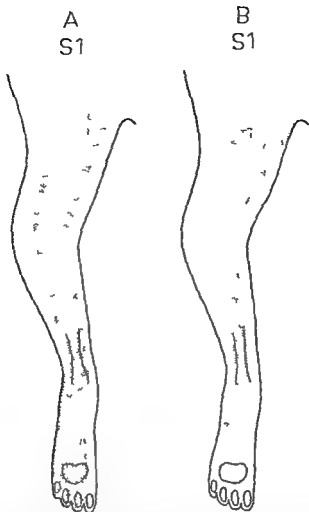


FIG. 4. Diagram showing the posterior aspect of the hindlimb with the S1 dermatome (dotted area) delineated by recordings from single units in the dorsal root (A) and from dorsal rootlets (B). N. tibialis and n. peroneus communis were sectioned.

### Discussion

Recordings from rootlets were made in the present work and also in Kuhn's study. The preparations used, however, were not quite comparable since in the present investigation the muscle nerves were sectioned. This was considered necessary in view of the fact that the muscle spindles are known to have a very low threshold to mechanical displacements. On the other hand, this means that some cutaneous branches were also cut which should cause some dermatomes to appear smaller than they actually are. With the procedure used here this applies especially to the area on

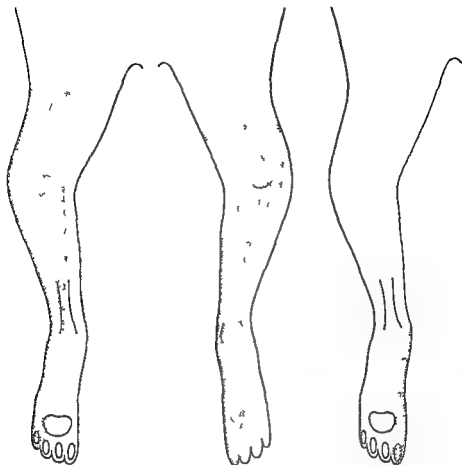
A  
L7B  
L6C  
L6

FIG 5 Same as Fig 4(A) The L7 dermatome on the posterior aspect of the hindlimb (A) the L6 dermatome on the anterior (B) and posterior (C) aspects of the limb

the foot By recording from rootlets, the activity in some few fibres may be shunted if the ratio of active to inactive fibres is small Since the same recording method was used in the present work as in *Kuhn's* study this cannot explain the difference in the results In addition single unit recording was used in the present investigation By this method the above mentioned error is excluded On the other hand findings from many animals have to be combined which due to the variation between specimens should tend to make the dermatomes larger than the actual one in each individual animal

The results obtained by mapping the L6 and L7 dermatomes were essen

tially the same as those of *Klessens* (1914). As compared with the L6 and L7 dermatomes found by *Kuhn* (1953) those found in the present investigation were somewhat narrower. This difference might be due to the absence of influences from muscle organs. The results obtained for the S1 dermatome however disagree with *Kuhn's* description but in the opposite direction to the discrepancies regarding L6 and L7 in a material of 4 animals. *Kuhn* showed that the tactile S1 dermatome innervates only the skin of the buttocks and the base of the tail. In the present investigation however, cutaneous units of the S1 dorsal root were also found regularly distally to the knee i.e. on the calf heel and proximal part of the foot and sometimes also in the distal part of the foot. This finding is essentially in agreement with the early work of *Klessens* (1914). The distribution of the S1 dermatome was also investigated by *Hagbarth* (1952) who studied the excitatory and inhibitory effect of skin pinching on the activity recorded from the nerve to the ankle extensors before and after sectioning the S1 dorsal root. He found that the region over the heel and the middle part of the plantar region were innervated by S1 (p. 43).

In this connection *Celestis* (1963) investigation of the segmental organization of the cortical afferent areas in the cat is of interest. On stimulating the S1 dorsal root he found that potentials were recorded not only on the medial aspect of the hemisphere where the buttocks and tail are represented but also on the dorsal aspects of the hemisphere in the hindlimb area. Assuming as claimed by *Kuhn* that S1 does not innervate the skin of the hindlimb he suggests that the latter response in the hindlimb area could have resulted from stimulation of fibres originating from the intrinsic muscle of the foot. In the light of the present investigation and those of *Klessens* (1914) and *Hagbarth* (1952) however the appearance of responses in the cortical hindlimb area on stimulation of the S1 dorsal root certainly need not be explained by activity in noncutaneous afferents.

### Summary

1) The tactile dermatomes L6—S1 in the cat were mapped by means of mechanical stimulation of the skin and a) single unit recordings in the dorsal root and b) recordings from dorsal rootlets.

2) The L6 and L7 dermatomes which are seen as longitudinal stripes on the hindlimb reaching down to the toes are situated on the antero-medial and postero-lateral aspects of the limb respectively (Fig. 5).

3) Somewhat more medially in relation to the L7 dermatome the S1 dermatome was situated on the posterior aspects of the limb usually reaching to the middle of the foot but sometimes down to the toes (Fig. 4).

## Results

### A ADULT CATS

For comparison the fibre composition of cutaneous and articular nerves in the adult stage is described before the postnatal development of such nerves. As can be seen in Fig 6 showing the calibre spectra of cutaneous and articular nerves in an adult cat, the largest fibres of the hindlimb nerves studied were found in nn cut fem lat saphenus and suralis. The largest fibres of nn plantaris and per superf were smaller than those of the three nerves mentioned above. The size of the largest fibres of n cut fem post was in between those of the largest fibres in the above two groups of nerves.

The cutaneous branch of n radialis taken in the forefoot contained nearly as large fibres as the nerve containing the largest fibres of the hindlimb.

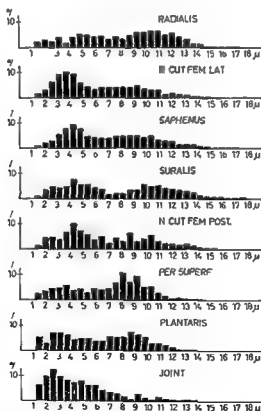


FIG 6 Histograms of calibre spectra of cutaneous nerves and the medial knee joint nerve in an adult cat. For further explanation see text.

The calibre of the largest fibres of the medial knee joint nerve was within the range of those of the hindlimb cutaneous nerves

The configuration of the histograms of the adult cutaneous nerve fibres can be seen in Fig. 6. Cutaneous nerves have two maxima. The absolute figures for these maxima and the largest fibres can be seen in Fig. 6 and in the Tables. An exact description of each spectrum was considered beyond the scope of this study. The configuration of the calibre spectra of the cutaneous nerves and that of the articular nerve might, however, be compared. It can be seen that the medial knee joint nerve had only one maximum (Skoglund 1956, Ekholm and Skoglund 1964a) whereas the cutaneous nerves had two maxima (Skoglund and Romero 1965).

## B. KITTENS

The myelinated fibres of the newborn kittens were very small in the cutaneous nerves. In fact, it was difficult to stain the thin myelin sheaths (cf. Skoglund and Romero 1965). Perfect sections of all nerves studied in the same kitten were not always obtained in the most immature cases.

The following text refers to Figs. 7—8 which are histograms of cutaneous and articular nerves of different maturation. Four nerves have been chosen for illustration.

1) N cut fem lat supplying a skin area on the thigh — a proximal structure (see Chapter II). This is one of the nerves containing the largest fibres in the adult cat.

2) N per superf supplying a skin area on the foot — a distal structure (see Chapter II). The largest fibres of this nerve in the adult cat were smaller than those of the other cutaneous nerves except for n plantaris which was similar to n per superf.

3) N radialis supplying a skin area on the forefoot (representing a forelimb nerve) and having nearly as large fibres as the largest ones of the adult hindlimb nerves studied.

4) The medial knee joint nerve (articular structure).

As can be seen in Fig. 7A from a 4 day old kitten the maximum of each calibre spectrum was found in the group of smallest fibres (between 1.21 and 1.76  $\mu$ ). N cut fem lat had a few (three) fibres that were larger than the largest ones of n per superf and the articular nerve. N radialis had a fibre still larger than the largest examples of n cut fem lat.

In Fig. 7B from a 15 day old kitten the same pattern as in Fig. 7A can be seen. N radialis had the largest fibres and n cut fem lat had fibres larger than the largest ones of n per superf and the articular nerve.

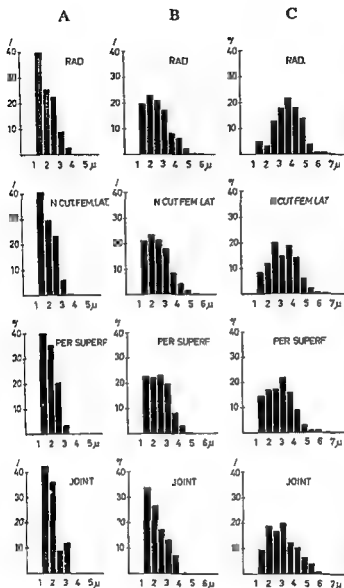


FIG 7 Histograms of calibre spectra of cutaneous nerves and the medial knee joint nerve in 4 (A) 15 (B) and 21 (C) days old kittens

As can be seen in Figs 7C and 8A C from kittens up to 90 days old, the same relations between the largest fibres of the nerves were essentially maintained in kittens up to 65 days old. In the 90 day old kitten (and in the adult stage Fig 6) however n cut fem lat had larger fibres than n radialis.

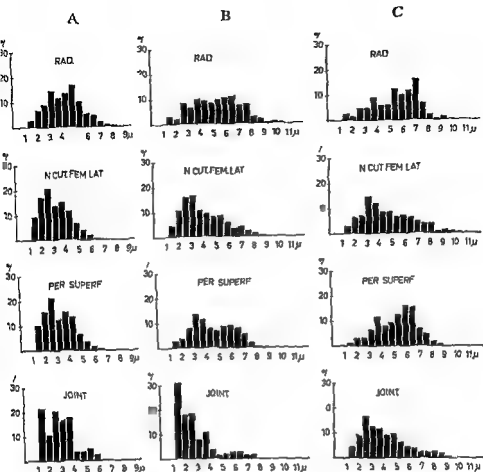


FIG 8 Histograms as in Fig 7 in 45 (A) 65 (B) and 90 (C) days old kittens

In Fig 9 the calibres of the largest fibres of these nerves have been plotted against age. It can be seen that in the beginning of the developmental stage up to an age of 65 days *n* radialis contained the largest fibres. After this age *n* cut fem lat had larger fibres than *n* radialis. It is also obvious that the largest fibres of the medial knee joint nerves never exceeded the diameter of the largest fibres of *n* cut fem lat. The calibres of the largest articular nerve fibres were about the same as *n* per superf or smaller.

In the 21 days old kitten (Fig 7C) and in younger kittens only one maximum appeared in each calibre spectrum. In the 45 days old kitten (Fig 8A) however two different maxima were discernible in some cutaneous nerve calibre spectra. The two maxima of cutaneous nerve calibre spectra were sometimes seen in the 65 days old and 90 days old kitten as well.



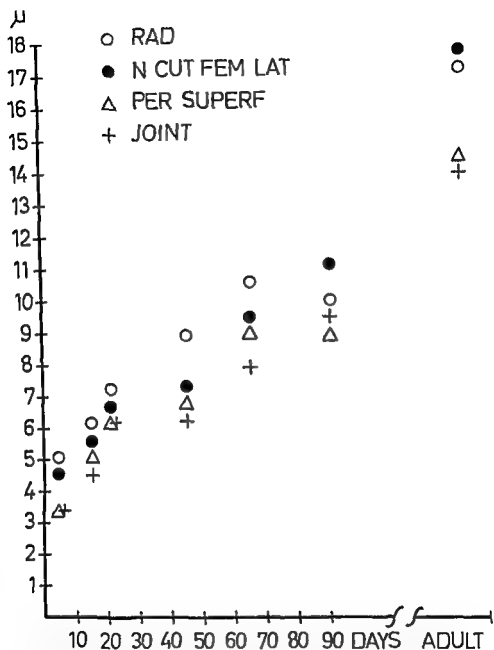


FIG 9 Largest fibre diameter of cutaneous and articular nerves plotted against age  
All nerves plotted on a particular age are obtained in one individual animal

as in the adult cat. The articular nerve on the other hand never developed two maxima in its fibre spectrum.

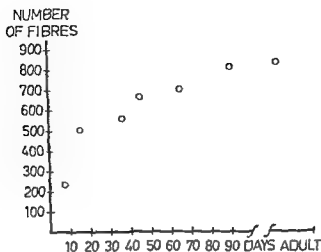


FIG. 10 The total number of myelinated fibres in n. suralis plotted against age

### C TOTAL NUMBER OF MYELINATED FIBRES

It has been pointed out earlier (Rexed 1944 Skoglund and Romero 1965) that the number of myelinated fibres increases during development. In the present study n. suralis was chosen for such an analysis. In Fig. 10 the total number of myelinated fibres in the sural nerve are plotted against age. The adult number of myelinated fibres was reached at an age of about 2–3 months.

### Discussion

Before discussing the comparisons between the calibre spectra, some comments on the method should be made. As has been pointed out earlier (Skoglund and Romero 1965) the correlation between chronological age and the actual maturity of the nervous system varies considerably between different kittens. Thus, it has to be taken into consideration that the fibre composition of comparable nerves and the size of the largest nerve fibres of such nerves in different animals of the same age varies over a rather wide range. It is evident that comparisons between calibre spectra within a series of animals must be made with certain precautions when each age is represented by only one animal. However, comparisons between different nerves within the same animal can be made with more certainty. The inter-individual differences are then no problem, but the variations in the methodological errors have to be considered. When comparisons with other investigations are made, methodological differences must be considered.

The technical quality of the material is of great importance, if the sections are bad satisfactory measurements cannot be made. This is a problem especially when using material from young animals. One of the efforts made here to obtain good technical quality, was fixation of the material in an isotonic buffer considered to give minimum deterioration of the structure (Sjostrand 1953). Furthermore all material not successfully treated was discarded. All measurements were made by one person (the author) and in accordance to certain rules (see Methods). When using this fixation a somewhat irregular form of cross sectioned nerve fibres is obtained. A relatively high primary magnification in the photomicroscope was used in order to obtain high quality photocopies of low secondary magnification.

Skoglund and Romero (1965) showed a proximo distal development of muscle nerves. For direct comparisons to be made, with any degree of accuracy, between different nerves with regard to their maturation at a certain developmental stage it seems to be a prerequisite that their largest fibre diameters either become eventually of the same order of size or that if they show an ultimate difference they will also show a difference in the opposite direction during some stage of the development. However comparisons can also be made between the relative stages of maturation of different nerves that is to say the number of times the different nerves have to increase their largest fibre diameter to reach their adult stage, regardless of whether they eventually become similar in size or different one way or the other.

One aim was to compare cutaneous nerves innervating proximal and distal structures directly e.g. *nn. cut. fem. lat.* and *per. superf.* The largest fibre diameters of these nerves differ during the postnatal development but they also differ in the same direction in the adult stage. Thus direct comparisons at different stages of development could not be made. A relative value of maturation was obtained however and it was found that in the 4 days old kitten the distal nerve (*nn. per. superf.*) had to increase its largest fibre diameter 4.23 times to reach the adult size whereas the proximal nerve (*nn. cut. fem. lat.*) in spite of the fact that it eventually reached larger diameters in the adult stage than the distal nerve only had to increase its largest diameter 3.92 times. In this sense the proximal nerve appears to be relatively more mature than the distal nerve just after birth. A similar calculation for the articular nerve gave an increase of 4.06 times. This nerve which supplies a different structure lying between that of the proximal and the distal cutaneous nerves thus shows a developmental value lying in between those for the cutaneous nerves. Using the material obtained by Skoglund and Romero (1965) the same calculation can be made for

n proximal (adductor) and a distal muscle nerve (gastrocnemius med.) The largest diameter of the distal nerve increases 4.35 times from the newborn to the adult stage whereas that of the proximal nerve increases only 4.06 times.

Thus the results obtained by comparisons of the calculated relative stages of maturation between proximal and distal nerves supplying different types of structures are consistent with the finding of a proximo-distal development in the muscle nerves (Skoglund and Romero 1965) obtained by means of direct comparisons.

The finding that n. radialis of the forelimb had larger fibres than any of the hindlimb nerves up to 65 days and that after this age the largest fibres were smaller than the largest fibres of the hindlimb nerves might indicate that the forelimb nerves are more highly developed at birth and for some time afterwards (cf. Skoglund and Romero 1965). This is in accordance with a cephalo-caudal development (Kingsbury 1932). N. radialis increases its largest diameter 3.39 times from the age of 4 days to the adult stage. This value is lower than those of the hindlimb nerves investigated here and indicates in accordance with the direct comparison that n. radialis is more mature than the hindlimb nerves just after birth.

The fibre analysis of the medial knee joint nerve during the postnatal development which has not been made previously, shows that the calibres of the largest fibres of most knee joint nerves were about the same as n. per. superf. The largest fibres of the joint nerves were never larger than those of the cutaneous nerves containing the largest fibres of the hindlimb. Skoglund (1956) showed that in adult cats there is normally a considerable variation in the sizes of the largest fibres of articular nerves. This is probably due to the different pathways available for the knee joint nerve fibres to reach the spinal cord: the medial nerve comprises only a part of the fibres.

In connection with physiological experiments on the knee joint in the cat (Ekholm and Skoglund 1960, Ekholm, Eklund and Skoglund 1960) the author has earlier studied the calibre spectra of adult medial knee joint nerves (Ekholm and Skoglund 1964a). The wide variation found by Skoglund (1956) was confirmed and it was shown that sympathetic nerve fibres probably do contribute but fairly little to the groups of small myelinated fibres of the knee joint nerve. The fibre spectrum of the adult medial knee joint nerve of the adult cat shown in the present investigation is in line with those of the nerves containing the relatively large fibres in the earlier investigation mentioned above. This fibre spectrum is thus suitable for comparison with the material from young kittens. The calibre spectra of n. saphenus and n. suralis were essentially in accordance with those obtained by Skoglund and Romero (1965).

Table I The calibre spectrum of n radialis

Animal No	121	176-	23	287	342	397	452	508	563	618	673	728	784	839	894	949	9
	176	232	287	34	397	452	508	563	618	673	728	784	839	894	949	9	
156	231	148	130	52	16	2	1										
130	175	152	159	66	14	5	1	1									
154	172	201	191	154	80	53	22	4	1								
196	26	17	65	91	111	93	71	20	6	8	1						
131	43	111	149	237	189	217	271	170	134	77	31	12	4	1			
201	25	13	52	41	63	58	53	61	66	69	46	48	18	12	3		
133	13	8	29	27	46	30	30	63	52	60	81	34	12	4	7		
149	14	19	18	30	14	31	39	36	31	32	25	30	25	36	46	4	

Table II The calibre spectrum of n cut fem lat

Animal No	11	176-	23	87	34	397	45	508-	563	618	673	778	784-	839	894-	949-	9
	176	232	287	343	397	452	508	563	618	673	778	784	839	894	949	9	
156	220	159	127	33	2	1											
130	182	156	126	102	58	4	4	6									
154	181	199	183	155	75	42	14	2									
196	62	88	148	114	138	106	46	18	8	2							
131	127	231	285	188	204	155	93	55	29	6	1						
201	28	68	102	106	67	57	53	55	37	22	24	15	8	3	1		
133	18	38	44	89	73	54	54	40	44	39	30	24	24	6	11		
149	29	56	120	175	191	172	112	84	61	78	77	77	78	82	90	6	

Table III The calibre spectrum of n saphenus

Animal No	1	176-	23	287	34	397	45-	508	563-	618	673	728-	784-	839	894-	949-
	176	23	287	34	397	45	508	563	618	673	728	784	839	894	949	
156	307	273	181	46	1											
130	44	115	151	100	40	5	1									
196	47	56	76	87	73	60	32	15	3							
131	251	313	373	261	254	276	263	161	107	41	14	4	1			
201	41	53	76	61	48	48	52	63	63	52	31	14	9	2	1	
133	20	27	45	81	57	58	53	75	62	46	26	28	9	13	4	
149	29	63	94	175	241	286	245	155	138	127	132	152	155	162	164	13

Table IV The calibre spectrum of n suralis

Animal No	1	1	176-	23-	87	34	397	45	508	563	618	673	728	784-	839	894	949	9
	176	1	287	34	397	45	508	563	618	673	728	784	839	894	949	9		
130	36	85	100	23	6	2	1											
154	147	105	107	74	47	21	6	1										
172	26	37	64	137	122	106	57	8	3	2								
131	54	80	104	88	82	108	75	41	23	7	4	1						
201	44	49	79	68	53	42	45	88	85	77	43	16	10	5				
133	11	30	67	77	99	63	69	87	88	69	71	37	14	14	6	3		
149	9	28	39	76	43	65	50	50	37	32	15	19	23	30	27	50		

04- 60	10 60- 11 15	11 15- 11 70	11 70- 12 25	12 25- 12 80	12 80- 13 36	13 36- 13 91	13 91- 14 46	14 46- 15 01	15 01- 15 56	15 56- 16 12	16 12- 16 67	16 67- 17 22	17 22- 17 77	Number of fibres measured
														580 573 878 507 1646 634 498 742
1 50	49	37	43	31	24	11	11	3	4	4	1	1		

04- 60	10 60- 11 15	11 15- 11 70	11 70- 12 25	12 25- 12 80	12 80- 13 36	13 36- 13 91	13 91- 14 46	14 46- 15 01	15 01- 15 56	15 56- 16 12	16 12- 16 67	16 67- 17 22	17 22- 17 77	Number of fibres measured
														542 638 851 730 1374 646 598 1830
3 61	1 65	32	36	25	23	11	12	5	6	1	0	1	2	

04- 60	10 60- 11 15	11 15- 11 70	11 70- 12 25	12 25- 12 80	12 80- 13 36	13 36- 13 91	13 91- 14 46	14 46- 15 01	15 01- 15 56	15 56- 16 12	16 12- 16 67	16 67- 17 22	17 22- 17 77	Number of fibres measured
														808 456 449 2319 614 607 3110
60	111	90	83	44	46	43	36	20	10	7	3	1		

04- 60	10 60- 11 15	11 15- 11 70	11 70- 12 25	12 25- 12 80	12 80- 13 36	13 36- 13 91	13 91- 14 46	14 46- 15 01	15 01- 15 56	15 56- 16 12	16 12- 16 67	16 67- 17 22	17 22- 17 77	Number of fibres measured
														233 233 560 667 704 813 829
1 44	45	38	34	28	26	21	11	8	6	6	1	1	7	

Table V The calibre spectrum of n per superl

Animal No	1	1	176	23	287	34	397	45	508	563	618	673	728	784	839	894	9
	176	3	287	34	397	-45	508	563	-618	-673	728	784	839	894	949	10	
156	256	226	133	22													
130	86	169	142	44	10												
154	215	218	220	187	78	29	2										
196	110	88	132	163	120	68	24	2		2							
131	130	204	278	163	198	176	85	44		23	4						
201	18	26	57	97	83	53	81	100		101	58	38	14	4	2		
133	7	19	20	39	70	49	56	75		98	93	39	27	9	3		
149	14	22	29	30	36	24	27	19		26	25	27	42	66	44	59	37

Table VI The calibre spectrum of n plantaris

Animal No	1	1	176	23	287	34	397	45	508	563	618	673	728	784	839	894	9
	176	3	87	342	397	-45	508	563	618	673	728	784	-839	894	949	10	
156	135	127	106	22													
154	290	222	233	213	133	63	9										
196	101	75	123	152	109	56	22	2		1							
201	51	49	43	57	50	57	91	99		71	36	8	3	2			
149	47	30	60	59	52	36	42	41		33	34	47	44	45	64	52	48

Table VII The calibre spectrum of n cut fem post

Animal No	1	1	176	23	287	34	397	45	508	563	618	673	728	784	839	894	9
	176	3	287	34	397	-45	508	563	618	673	728	784	839	894	949	10	
156	49	41	32	4													
201	26	27	70	60	40	27	39	59		44	34	16	4	2	1		
149	8	26	29	22	41	61	44	35		23	29	14	25	22	40	19	25

Table VIII The calibre spectrum of the medial knee joint nerve

Animal No	1	21	176	23	87	34	397	45	508	563	618	673	728	784	839	894	9
	176	t	■	347	397	-452	508	563	-618	-673	728	784	839	894	949	10	
185	12	11	10	10													
156	14	12	3	4													
199	14	6	4	11	2	1											
182	14	12	24	12	2	6											
154	33	76	17	13	7	1											
196	9	17	16	19	12	10	6	4		1							
179	10	27	17	26	18	12	3	8		3							
172	7	9	10	7	5	6	4	8		1							
131	36	18	34	28	29	6	6	8		4							
201	45	26	27	11	16	5	2	3		4	4	2	2				
200	5	5	11	14	17	21	17	10		9	8	4	3	6	11		
133	8	17	31	27	22	17	18	12		9	7	4	4	5	2	1	
149	18	28	37	29	24	22	23	19		19	13	10	11	6	2	9	4

10 60-	11 15-	11 70-	12 25	12 80	13 36-	13 91	14 46-	15 01	15 56-	16 12	16 67	17 22	Number of fibres measured
11 15	11 70	12 25	12 80	13 36	13 91	14 46	15 01	15 56	16 12	16 67	17 22	17 77	
													637
													451
													949
													752
													1305
													732
													604
													600
20	11	4	5	2	1	1							

10 60	11 15-	11 70-	12 25	12 80-	13 36-	13 91	14 46-	15 01	15 56	16 12	16 67	17 22	Number of fibres measured
11 15	11 70	12 25	12 80	13 36	13 91	14 46	15 01	15 56	16 12	16 67	17 22	17 77	
													390
													1163
													641
													617
													825
26	23	11	3	1	1								

10 60-	11 15	11 70-	12 25	12 80	13 36	13 91	14 46-	15 01	15 56-	16 12	16 67	17 22	Number of fibres measured
11 15	11 70	12 25	12 80	13 36	13 91	14 46	15 01	15 56	16 12	16 67	17 22	17 77	
													126
													449
													590
19	16	13	12	14	9	5	2	1					

10 60	11 15	11 70-	12 25	12 80	13 36-	13 91	14 46	15 01	15 56-	16 12	16 67	17 22	Number of fibres measured
11 15	11 70	12 25	12 80	13 36	13 91	14 46	15 01	15 56	16 12	16 67	17 22	17 77	
													43
													33
													26
													70
													97
													95
													124
													57
													169
													147
													130
													184
													288
5	3	2	1	2	1								



## Summary

1) The postnatal development of the fibre composition (calibre spectra) of cutaneous and articular nerves was studied in osmium stained sectioned material. For comparison data from adult material was also presented.

2) In newly born kittens the myelinated fibres of cutaneous nerves were very small. The diameter range of the nerve fibres increases during the postnatal development and the increase in largest fibres at an age of 90 days was about 40—50 % of the total increase between birth and the adult stage.

3) The following relationships between the largest fibres in some of the cutaneous nerves studied were maintained up to an age of 65 days. N. radialis (forefoot) contained the largest fibres. n. cut. fem. lat. (proximal structure of hindlimb) had larger fibres than n. per. superf. (distal structure of hindlimb). At a later age and in the adult stage n. cut. fem. lat. had larger fibres than n. radialis. The change of the relationship between the forelimb and hindlimb nerves is consistent with a cephalo-caudal development and calculation of the relative maturation of proximal and distal cutaneous hindlimb nerves gave values consistent with a proximo-distal development.

4) The size of the largest fibres of the medial knee joint nerve was about the same as that of fibres in n. per. superf. and never exceeded the largest fibres of n. cut. fem. lat.

## CONDUCTION VELOCITY OF CUTANEOUS NERVES DURING POSTNATAL DEVELOPMENT

### *Introduction and literature*

A relationship between conduction velocity and fibre diameter was reported by Hursh (1939 a) — the well known factor of six Hursh (1939 b) also studied the properties of growing nerve fibres in cats and kittens. The conduction velocity of cutaneous nerves (n. saphenus) increased from 11 m/sec at an age of 4 days to 60 m/sec at 87 days. The increasing conduction velocities were plotted against the increasing length of the leg during the development. The relationship was clearly a linear one. Hursh concluded that the movements of the kittens are not slowed because of the lower conduction rate since the latter is nicely compensated for by the decreased length of the nerve path.

As pointed out by Skoglund (1960 b) this conclusion cannot be drawn from Hursh's material. The conduction time for a stretch of nerve of the same length as the limb can actually be calculated from Hursh's material to decrease with increasing length of the limb. As for muscle afferents Skoglund (1960 b) calculated that 80 % of the decrease in conduction time has already occurred when the conduction velocity has reached 30 m/sec (at an age of about 20 days). For comparison with the muscle afferents he also presented some conduction velocity values from the sural nerve and showed that here the conduction velocity increased more slowly than that of the muscle nerves (0.65 m/sec per day compared with 0.99 m/sec per day).

In the present work the postnatal changes in the conduction velocity of cutaneous nerves have been studied with special regard to the following relationships

- 1) Conduction velocity — chronological age
- 2a) Conduction velocity — increase in length of limb
- 2b) Conduction time — increase in length of limb
- 3) Conduction velocity — fibre spectra (Chapter V)

## Material and Methods

The conduction velocity of the sural nerve was usually determined in connection with other physiological experiments. A large number of values were obtained from kittens and cats ranging in age from newborn to adult. Pentobarbital was used for anaesthesia. A lumbo sacral laminectomy was performed and the dorsal roots L6, L7 and S1 were freed and sectioned near the spinal cord (for details of the method see Chapter III). The sural nerve was stimulated with squarewave shocks having a duration of 0.3 msec and a strength supramaximal to the first potential wave recorded. The potentials were recorded from one of the appropriate dorsal roots (see Chapter III) and photographed on bromide paper.

The conduction velocity of the sural nerve was calculated in the following way. The conduction time was measured on the bromide paper from the beginning of the shock artifact to the rise of the first potential wave. The conduction distance was measured from the cathode of the stimulating electrode to the recording electrode on the dissected nerve. In some cases the same nerve in which the conduction velocity had been determined was taken out for staining and fibre analysis (Chapter V). Postmortally the maximal length of the femur was measured from the top of the femoral head to the distal end of the condyles.

## Results

In Fig. 11 the conduction velocity of the sural nerve is plotted against age. It can be seen that in newborn kittens the conduction velocity varied between 8–12 m/sec and increased with increasing age by approximately 0.6 m/sec per day. The equation of the linear regression line calculated by the method of least squares is  $y = 0.58x + 10.9$ . This value is similar to though slightly smaller than that (0.65 m/sec per day) given by Skoglund (1960 b).

The conduction velocity was then plotted against the length of the femur (Fig. 12). The linear regression equation (method of least squares) is  $y = 1.03x - 14.2$ . It shows the trend of the development which may be expressed as follows. The conduction velocity approximately increases by 1 m/sec per 1 mm increase in the length of the femur from 10 m/sec at 25 mm femur in the newborn stage (up to a stage when the femur is 60 mm long).

The conduction time for a stretch of nerve of the same length as the femur was calculated from the regression line in Fig. 12 and plotted in Fig. 13. When the femur was 25 mm the conduction velocity was 10.8

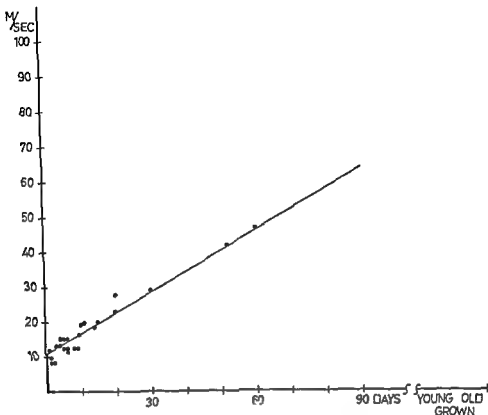


FIG. 11 Conduction velocity of *n. suralis* plotted against age See text

m/sec and the conduction time 2.31 msec whereas the conduction time was 1.37 msec at a stage when the femur was 60 mm and the conduction velocity 45 m/sec. It can be seen that the conduction time decreases with increasing femur length.

The graph shown in Fig. 12 and the linear regression line can be used when a rough estimate of the conduction velocity is desired without the use of electrophysiological measurements. For practical purposes a simple formula was calculated. Since the slope is very near unity only one constant has to be used ( $y = x - 14$ ). It follows that the length of the femur expressed in mm minus 14 gives the numerical value of the conduction velocity of the sural nerve (m/sec) with an accuracy of about  $\pm 5$  m/sec (this error was not calculated statistically but estimated by eye).

For purely practical purposes a graph (Fig. 14) is shown in which the length of the femur is plotted against age. This graph can be used when it is desired to estimate the age of the animal.

M/SEC

100

90

80

70

60

50

40

30

20

10

10

20

30

FEMUR LENGTH MM

85-100

ADULT

FIG. 12 Conduction velocity of n. suralis plotted against femur length. See text

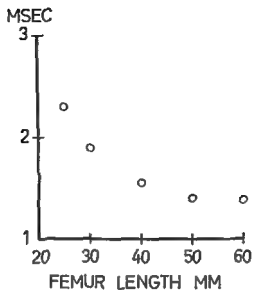


FIG 13 Conduction time for a stretch of nerve (suralis) of the same length as the femur plotted against femur length

FEMUR LENGTH

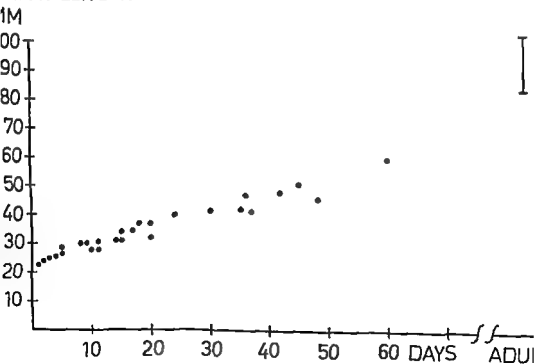


FIG 14 Length of femur plotted against age

Table IV Quotient between conduction velocity in the largest fibres and fibre diameter in *n. suralis*

Animal No	Conduction velocity m/sec	Largest fibre diameter ( )	Quotient cond vel /diam
1	22.0	4.52	4.9
2	22.8	5.08	4.5
3	29.3	6.18	4.7
4	29.5	6.73	4.4
5	39.5	7.84	5.0
average			4.7

The relationship between conduction velocity and fibre size has been investigated earlier experimentally. Since, however, the experimental conditions may vary from one investigation to another, it cannot be expected that the relationship found here is strictly the same as those obtained in other experiments. In order to provide a rough check of the method used in the present investigation a fibre analysis was made on the same sural nerve in which the conduction velocity was determined. In Table IX it can be seen that when the conduction velocities are divided by the maximum fibre diameter of the calibre spectrum (largest diameter of the group of largest fibres) factors are obtained varying from 4.4—5.0, with an average of 4.7. This value can be used for a rough calculation of the fibre spectrum corresponding to a certain conduction velocity (see Discussion).

## Discussion

The postnatal increase of 0.6 m/sec per day in the conduction velocity of the sural nerve found in this study is consistent with but somewhat smaller than that observed by Skoglund (1960 b).

The conduction velocity has been correlated with the femoral increase and it was found that the velocity increased by approximately 1 m/sec per 1 mm increase of the femur. The calculated conduction time for the stretch of nerve of equal length to the femur decreases with increasing conduction velocity and increasing femoral length. It follows that in young animals the slow conduction is not completely compensated by the shorter impulse pathway. This confirms the calculations made by Skoglund (1960 b) but disagrees with the conclusion drawn by Hursh (1939 b).

In laboratory work with kittens it is often a problem to determine the

age of the animal and its degree of nervous maturation *Skoglund* (1960 ■ c) used conduction velocity as a measure of nervous maturity. The gestation period ■ reported by *Scott et al* (1957) to be  $65 \pm 4$  days. In the present investigation a graph and ■ simple formula ( $y=x-14$ ) are given by which a rough calculation of the conduction velocity in the sural nerve can be made from ■ knowledge of the length of the femur. Another graph ■ given from which the age of the animal (if unknown) can be calculated from the length of the femur.

Two regression lines have been calculated. It may be argued that the best fit of the values is not given by a straight line. In fact the values presented in Fig. 12 give the impression of a somewhat curvilinear relationship. The straight regression lines, however, have been given to illustrate the general trend of the development. Although they may not be those best fitted to the experimental values the conclusions drawn from them seem valid.

The ratio between conduction velocity and fibre diameter was 4.7. This figure is obviously restricted to the experimental conditions of the present investigation. Several possible errors are involved. The conduction time is measured to the rise of the first potential wave. This point on the neurogram is easily distinguishable and offers a stable and clear point for measurements. A few very large fibres (see Chapter V) may cause an early deflection of the neurogram, but this deflection is ill defined and thus not suitable for measurements. The method for measuring time used here thus gives lower values for conduction velocity than the true values. When evaluating the ratio between conduction velocity and fibre size several other factors must also be taken into consideration, e.g. the actual point of stimulation, the measurement of the length of the dissected nerve, the temperature and the morphological technique.

The correlation factor obtained here (4.7) ■ lower than the value of six given by *Hursh* (1939 a). The regression line calculated from *Hursh's* (1939 a) material has a slope of 6.0 which means that the velocity in meters per second can be obtained by multiplying the diameter in micra by this factor. However *Hursh's* relationship appears to be expressed by the following equation:

$$\text{conduction velocity} = 6 \times \text{fibre diameter} - 4 \quad (1)$$

Thus simply multiplying fibre diameter by the factor of six should give an estimate of the conduction velocity which is 4 m/sec too high. The fibre diameter can be calculated from the conduction velocity since from (1)

$$\text{fibre diameter} = \frac{\text{conduction velocity} + 4}{6} \quad (2)$$



Again if the constant (4) is omitted an error is introduced which is proportionally greatest in the small diameters

In *Hursh's* material the conduction velocity for fibres  $4\ \mu$  and  $8\ \mu$  in diameter were 20 and 42 m/sec respectively i.e. the velocity diameter ratio was about 5 This is in fairly good agreement with the mean value of 4.7 obtained in the present investigation and seems to be a more correct factor than that suggested by *Hursh* (1939 a) especially for low conduction velocities

As will be further pointed out in chapter VII the stage at which the conduction velocity is 20 m/sec is of particular interest With the aid of the factor 4.7 the diameters of the largest fibres of such nerves can be calculated to be about  $4.3\ \mu$  If this diameter is compared with those of the calibre spectra of the sural nerves in the 14 and 21 day old kittens (the age at which the conduction velocity of 20 m/sec is attained) given in chapter V, it can be seen that these nerves have a few somewhat larger fibres but that there are a considerable number of fibres of about 4.3 microns The 45 day stage is also of interest with regard to the postnatal changes in the physiological properties of the cutaneous nerve fibres (Chapter VII) The conduction velocity is then about 37 m/sec The calculated diameter of the largest fibres is about  $8\ \mu$ , which fits well with the findings in Chapter V

### Summary

- 1) The conduction velocity of the sural nerve was determined in kittens of different ages

- 2) The conduction velocity increased by approximately 0.6 m/sec per day from approximately 10 m/sec at birth

- 3) The conduction velocity increased by approximately 1 m/sec per one mm increase of the femur postnatally

- 4) The conduction time for a stretch of nerve equal to the length of femur decreased postnatally

- 5) For practical purposes a formula was calculated which can be used for a rough estimation of the conduction velocity (in m suralis) from the femur length. conduction velocity (m/sec) = length of femur (mm) minus 14

- 6) When the conduction velocity (m/sec) of some sural nerves was divided by the diameter ( $d$ ) of the largest fibres of the calibre spectra obtained from the same nerves an average quotient of 4.7 was obtained

- 7) Using *Hursh's* (1939 a) own graph it was found that the conduction velocity cannot be obtained correctly by only multiplying the fibre diameter

with the factor of 6. The equation should be: conduction velocity (m/sec)  
 $\approx 6 \times \text{fibre diameter } (\mu) - 4$ . When the factor of 6 alone is used the  
 error introduced is greatest for small fibres. When calculating from *Hursh's*  
 data the factor for small fibres should be about 5.

8) The largest fibre of a nerve giving a conduction velocity of 20 m/sec  
 was calculated to be  $4.3 \mu$  by means of the factor 4.7 (the corrected *Hursh*  
 equation gives  $4 \mu$ ).

## CHAPTER VII

# DISCHARGE PATTERNS OF CUTANEOUS AND ARTICULAR SENSE ORGANS DURING POSTNATAL DEVELOPMENT

### *Introduction and literature*

Nerves course beneath the (superficial) epithelium and end under it in primitive free terminations before any reflexes can be elicited in mammalian embryos (Windle 1940). A morphological basis for cutaneous nerve activity is evidently established early in development. The function of skin afferents during ontogenesis has been studied in embryos and fetuses (for ref. see Windle 1940, Carmichael 1951, 1954). In all these studies the following procedure was used: the skin was stimulated and it was observed at what age a reflex movement appeared. The test situation used thus requires function in all parts of the reflex arc and does not give information about the sequence in which different parts of the reflex arc develop. However, since reflex movements are elicitable by mechanical (and thermal) stimulation of the skin in cat embryos (Windle and Griffin 1931) the sensory part of the reflex arc must function early in development.

The sensory areas or reflexogenous zones in the skin appear first in the head, neck and forelimbs and extend caudally with increasing age. The last such zones to be established are those of the hindlimb and tail, which however seem to exist before full term in kittens (Windle 1940). It should thus be possible to evoke activity from the cutaneous sense organs in these regions already at birth.

In this investigation the activity arising from the sense organs in the skin has been recorded from both peripheral nerves and single functioning dorsal root fibres in an attempt to analyze the discharge pattern during postnatal development. For comparisons with the exteroceptors of the skin the development of the discharge pattern in joint proprioceptors was also studied.

The activity of proprioceptors in kittens was studied by Skoglund (1960, 1963) who found that the discharge of receptors in the gastrocnemius muscle in response to stretch was phasic in newborn and young kittens. Tonic responses were obtained from proximal muscles at the same age. With increasing age more sustained discharges were obtained from the gastrocne-

mus muscle but the frequency of the discharge was low. The responses of Golgi tendon organs were also phasic in newborn kittens. At the stage when the muscle nerves have reached a conduction velocity of 20 m/sec and the absolute refractory period has attained adult values, tonic discharges from the muscle receptors were found. It was suggested that the appearance of tonic discharges might be related to parallel changes in the electrical properties of the nerve fibres and the mechanical properties of the muscle.

There is a complete lack of knowledge as regards the development of the discharge patterns from cutaneous afferents. The aim of the present investigation was twofold: 1) to ascertain if there is the same relation between discharge pattern and conduction velocity in the exteroceptors in the skin as in the proprioceptors; 2) to study the development of the discharge patterns of different types of cutaneous receptors to obtain the information necessary for an evaluation of the afferent link of the reflexes that can be elicited from the skin. Some of the findings reported here have been published in a preliminary note (Eklholm 1965).

To compare the properties of immature and mature sense organs, earlier experiments on sense organs in the adult cat have been repeated and in some respects extended. There have been numerous investigations since Adrian and Zotterman (1926a, b) succeeded in obtaining recordings from afferent nerve fibres and describing the discharge characteristics of individual sense organs. Much information is now available concerning different types of cutaneous afferents (for ref. see Granit 1955, Hensel 1966 a, b, Iggo 1966). Earlier observations on cutaneous sensory units in adult cats have been summarized in connection with the description of the present results in the immature animals. Joint proprioceptors of adult cats have been studied by Andrew and Dodd (1953), Boyd and Roberts (1953), Boyd (1953, 1954), Skoglund (1956) and Eklund and Skoglund (1960).

In the present investigation of the postnatal development of cutaneous sense organs, two types of cutaneous myelinated afferent units of hairy skin have been studied: 1) hair follicle afferent units (hair units); 2) slowly adapting mechano sensitive afferent units (touch units) (Iggo 1966).

The following specific questions have been treated: 1) Is the activity of the sense organs in young animals similar to that in adults? 2) If not, in what way does it differ? 3) What is the frequency-adaptation number of impulses etc. obtained in response to adequate stimulation of the immature sense organs? 4) Can such parameters be correlated to the maturity of the nerve fibres in terms of conduction velocities? 5) Are there differences in the development of the function of exteroceptors in the skin and the proprioceptors in muscles and joints?

## Material and Methods

The results from 95 cats and kittens anaesthetized with pentobarbital (40 mg/bw i.p.) are presented here. Usually the hairs of the hindlimbs were clipped down to a height of about 1 mm. After a lumbo sacral laminectomy the dorsal roots L4, L5, L6, L7 and S1 were dissected free and cut near the spinal cord. To avoid interference with activity from muscle and tendon sense organs the following muscle nerves were cut in the hindlimb: the hamstring, tibial, peroneal and femoral (except the saphenus). An afferent unit consists of a nerve fibre, its terminals and associated non-nervous elements (Iggo 1966). The fibres of the units were isolated in the dorsal root by dissecting free thin filaments under the dissecting microscope. Because of the size of the fibres this was a difficult and time-consuming work. Usually recordings were made from filaments containing only a single active cutaneous afferent but sometimes filaments containing 2—3 afferents were used. Sometimes only very few single units could be analyzed in the young animal.

In another type of experiment activity in a peripheral nerve was recorded either from the whole nerve or from a dissected thin strand of it. N. suralis was often used; this was dissected free for as long a stretch as possible in the popliteal fossa. The medial knee joint nerve (Gardner 1944; Skoglund 1956) was used for recording from joint sense organs.

Mechanical stimulation of the skin was performed with the fingers, a pair of forceps, a sable brush or a probe. In many experiments on the slowly adapting units constant stimulation was performed by applying a spring clip over the receptive skin area. In order to produce constant stimuli of different intensity strong and weak clips were used the same strong clip and the same weak clip being used in all the animals which were stimulated in this way.

Electrical stimulation was applied by a pair of needles inserted in the skin over the receptive field. Although not proved it is highly probable that in this case the nerve fibres or their terminations are stimulated directly. The duration of the impulses was 0.1 msec delivered at different frequencies in short trains (up to 10 sec) or for a longer period of time. This method is of course liable to error especially if threshold stimuli are used since the stimulation may cause large alterations in the skin resistance.

Temperature changes were induced by cooling with ice ether or ethyl chloride and by warming with a lamp. The knee joint receptors were stimulated by passive extension and flexion or by a probe pressed against the joint capsule.

For temperature control and protection of exposed tissue references should be made to Chapter III. The fixation of the animals was generally achieved by threads attached to the skin and a clamp around the base of the tail leaving one hindlimb completely free for localization, identification and stimulation of the receptive fields in the skin.

The position and shape of the receptive fields of the units were mapped directly on the skin. The area of the receptive field of a hair unit was calculated from two diameters measured at right angles ( $r_1 \times r_2 \times \pi$ ).

*Recording.* A nerve filament or a whole nerve was put on a silver hook; a reference electrode was inserted into inactive tissue. Different amplification systems (see Chapter III) with monitoring by both loudspeakers and oscilloscopes were used. The activity was photographed on moving bromide paper either with the oscilloscope beam sweep disconnected and with the camera at maximal speed or with sweep displays. To be able to resolve nerve spikes of high frequency it was sometimes necessary to feed the record into a high speed tape (Revox tape recorder). The tape was afterwards played back at low speed and the record displayed on an oscilloscope and photographed. A sine wave generator or a piezo-crystal indicated time on a second oscilloscope beam.

When measuring the maximal frequencies obtained in response to mechanical stimulation of touch units the number of spikes per period of time (50 msec) was counted; during this period more spikes were produced in the adult cats and older kittens (because of their high frequencies) than in young kittens. Since this might introduce an error when estimating the frequency the minimum interval between two spikes in the spike train was also measured. This gives a more exact value of the frequency over a short interval. The adapted frequency was measured as number of spikes per 1 sec.

In a series of experiments the conduction velocity of each isolated single unit was determined. Only units with their receptive fields in the sural area were used. Stimulation electrodes were applied to the undivided sural nerve in the popliteal fossa. This procedure however was found to damage the nerve fibres in the young kittens and many units were lost. Therefore in most cases the conduction velocity of the whole sural nerve was determined at the end of the experiment (see Chapter VI) and/or a specimen of the sural nerve was removed for fibre analysis (see Chapter V).

## *Results*

### A RECORDING FROM THE SURAL NERVE

Recordings were made from the sural nerve in the popliteal fossa, the

RAPID

SLOW

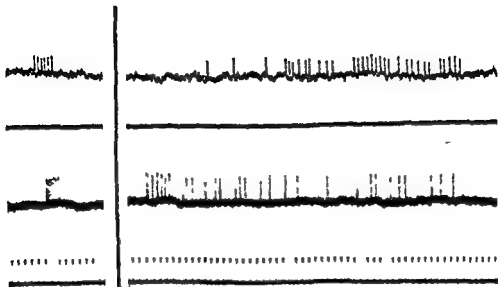


FIG. 16 Recordings from single hair units in response to rapid and slow strokes of a brush over the receptive fields in (A) 5 and (B) 45 days old kittens (Spikes retouched) Time 10 msec

kittens No attempt was made to study further the adaptation of the hair units in the kittens When the hairs within the receptive fields were moved the unit responded by a train of impulses The spike frequency in the train on the other hand was studied

*Mean frequency of impulse train* The hairs over a receptive field were bent by the stroke of a brush and the unit responded with a train of impulses As illustrated in Fig 16 the mean frequency of the train was lower and the duration of the discharge longer (the number of impulses greater) when the stroke was slow, than if the stroke was rapid By increasing the rapidity of the stimulus the mean frequency could be increased but only up to a certain limit which depended on the age of the animal as is also illustrated in Fig 17 The highest mean frequencies obtained in a series of stimulations with rapid strokes over the receptive fields were plotted as a function of age The unit that was found to give the highest mean frequency in each animal was selected In Fig 17 it can be seen that the maximal mean frequency of the hair unit response is low in young kittens and increases with increasing age to reach values in the adult range some 6 weeks after birth In order to include also

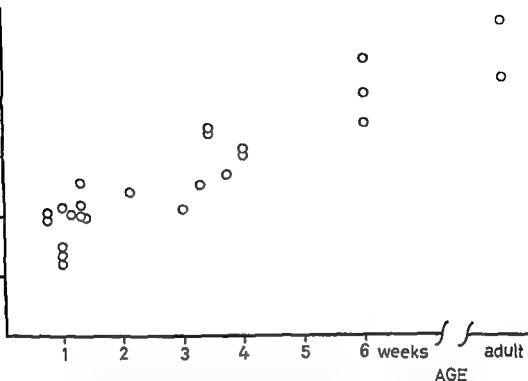


FIG 17 The highest mean frequency recorded from single hair units in response to rapid strokes of a brush over the receptive field plotted against age. The unit that was found to give the highest mean frequency (in spike trains of  $> 3$  spikes) in each animal was selected.

responses of only 2 spikes in the material the minimum interval found between 2 spikes regardless of the number of spikes in the response was plotted against age (Fig 18). The same trend as in Fig 17 was obtained. The interval was of relatively long duration in the early ages and decreased with increasing age to attain adult values at about 6 weeks.

The results show that in immature hair follicle units the capacity to produce high frequency responses is limited and that this capacity increases with increasing age.

*Electrical stimulation* When electrically stimulating the receptive fields it was found that the maximal frequency at which a one to one stimulus response relation was maintained was low in young kittens and increased with increasing age.

*Repeated stimulation* Attempts were made to ascertain whether the response of hair units changed when they were stimulated repeatedly by



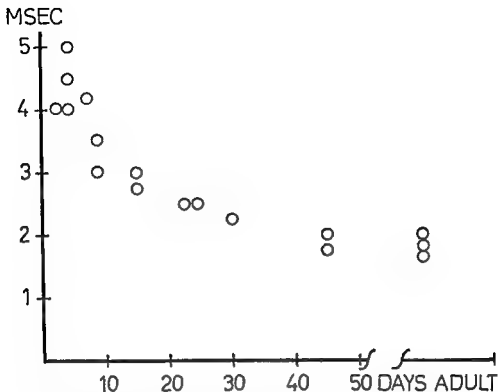


FIG 18 The minimum interval between two spikes recorded from single hair units in response to rapid strokes of a brush over the receptive field plotted against age. The unit that gave the shortest interval (regardless of the number of spikes in the spike train) in each animal was selected.

frequent rapid strokes of a brush. A receptive field was stimulated manually in this way as frequently as possible during one minute. The response was not apparently altered during the period of repeated stimulation; the frequency and number of impulses were essentially unchanged both in cats and in kittens. Thus, tested with this simple method, the ability of the hair units to respond repeatedly with a train of impulses at the frequency characteristic for the particular age of the animal seemed to be the same in both adult cats and kittens, but the period of repeated stimulation was probably too short to reveal any differences between immature and mature units.

*Receptive fields* The hair units respond to movements of the hairs only within a circumscribed area, the receptive field of the unit. When the present study was started it was found that the data on the receptive fields of the hair units in adult cats were incomplete. A comparison of

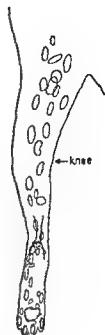


FIG. 19 Diagram showing the shapes of the receptive fields of hair units

the size, form and localization of such receptive fields was therefore undertaken in both adult cats and kittens. Such a study is also of interest for the comparison between the receptive fields of peripheral and central neurons.

The shape of the receptive fields of some adult hair units is illustrated in Fig. 19. Out of 80 units that were studied in this way, 83.7% had approximately oval receptive fields, usually with the long axis along the limb; 12.5% of the receptive fields were circular (10% situated on the foot and distal part of the lower limb and the remaining 2.5% more proximally on the limb). In a few cases (3.8%) the shape was irregular. Overlapping units were frequently found.

In Fig. 20 the surface area of the receptive fields of hair units in 72 adult (filled circles) and 36 non-adult (open circles) (less than 30 days old) animals has been plotted against the actual position on the limb, since all hindlimbs are not of equal length, plotting against a unit length would be erroneous. The sketch of the hindlimb serves as the abscissa against which the receptive areas have been plotted (actual longitudinal position). The range of the areas of the adult receptive fields is as seen in the Figure, very great, and exhibits a progressive increase in a proximal direction. In the adult animals, areas of greater than 150 mm<sup>2</sup> were not observed on the foot and

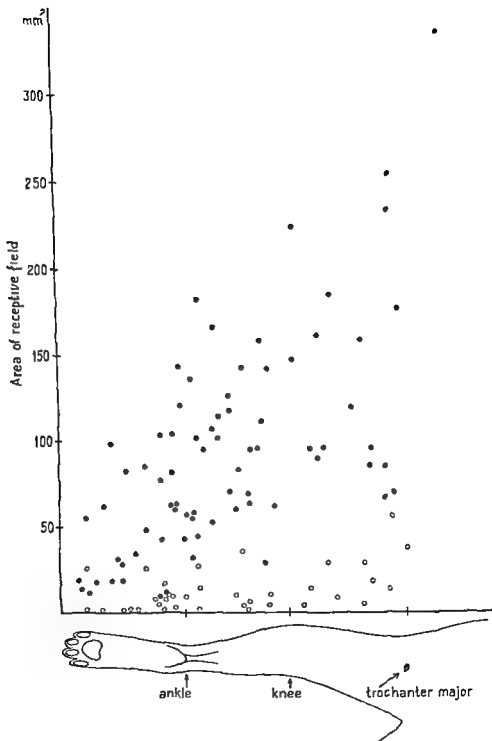


FIG. 20 The surface area of the receptive fields of hair units plotted against actual longitudinal position on the limb. The sketch of the hindlimb serves as abscissa. Filled

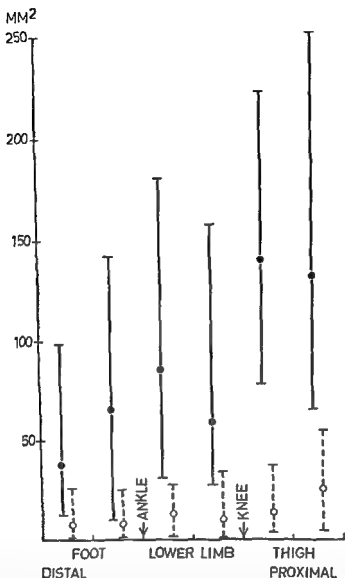


FIG. 21 The average and range of the surface area of the receptive fields of hair units localized in six limb segments. For further explanation see text. Continuous line and filled circles: adult units. Broken lines and open circles: non adult units (< 30 days).

there were no areas less than 50 mm<sup>2</sup> on the thigh. The non adult values were mainly in the low range and below the adult range.

The foot, lower limb and thigh were each divided transversely through their midpoints into proximal and distal parts. Thus in all there were six

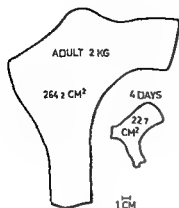


FIG. 22 Total hindlimb skin area in a 4 days old kitten and an adult cat

limb segments. The average and range of the receptive fields situated in each such segment was calculated and plotted in Fig. 21. As can be seen the average area of the adult receptive fields was 37.5 mm<sup>2</sup> on the distal foot whereas the average area was 131.6 mm<sup>2</sup> on the proximal thigh. The ratio between the average area in the proximal thigh and the distal foot was thus 3.5. In the kittens the average area of the receptive fields was 7.0 mm<sup>2</sup> on the distal foot and 25.5 mm<sup>2</sup> on the proximal thigh.

The skin area also increases during growth. As shown in Fig. 22, the total skin area of the hindlimb increases about tenfold between the age of 4 days and the adult stage.

Because of the technical difficulties only a few single units could be completely analyzed in each young kitten. Therefore the non adult material is smaller than that required for a strict statistical comparison with the adult material. However, large receptive fields were never seen in young kittens. The receptive areas were small in the young kittens and indeed often too small to be accurately measured with the method used. The shapes of the receptive fields in kittens appeared to be essentially the same as in adult cats but the proportion of round receptive fields might be somewhat greater than in the adult material. The real occurrence of overlapping receptive fields in the young kittens was also difficult to establish. Overlapping receptive fields were very seldom seen in young kittens but this may have been due to the small number of units studied in each animal.

## C THERMOSENSITIVITY OF AFFERENT UNITS

### 1 Earlier observations

The temperature sensitivity of afferent units in the hindlimb of adult cats has been studied by Witt and Hensel (1959), Hunt and McIntyre (1960 a)

and Iggo (1963 a) It has been found that many of the mechano receptors of the hindlimb skin are also sensitive to changes of the temperature, usually the units are excited by a sudden fall of the temperature and inhibited by sudden warming Some of these units have been shown to give a frequency maximum at a certain constant temperature level The temperature range associated with discharge is approximately 20—45°C

## 2 Present observations

The effect of sudden cooling was studied by recording from thin dorsal root filaments in kittens of different ages In the newborn animals sudden cooling usually gave rise to a discharge of relatively short duration This response could be produced in filaments that had no spontaneous activity before and after cooling but the fibres were sensitive also to mechanical stimuli Spontaneously firing units were also observed in the newborn kittens but were less frequent than in older kittens and adult cats Spontaneously firing units were not seen in recordings from the whole sural nerve in young kittens This is probably due to the shortcomings of the technique and the paucity of such units as compared with older kittens The spontaneously firing single units tested in newborn kittens usually responded on sudden cooling with a sudden frequency increase which then reverted to about the initial level Some of these units were tested also at different constant temperatures It was found that such units had frequency maxima at certain temperature levels as in adult cats Such temperature curves from

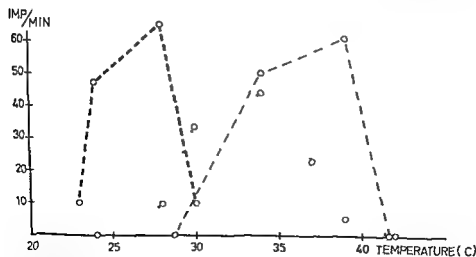


FIG. 23. 5 days old kitten. Steady discharge frequency (imp/min) of three mechanosensitive afferent units plotted against constant skin temperature (°C).

a 5 day old kitten are shown in Fig 23 These units were also sensitive to mechanical stimulation of the skin

In the newborn kittens there are thus, units that respond to sudden cooling and to different constant temperature levels The findings obtained in older kittens do not differ essentially from those of the young kittens except for higher frequencies and the more frequent occurrence of spontaneously firing units

## D TOUCH UNITS IN HAIRY SKIN

### 1 *Earlier observations on adult units*

Slowly adapting mechano sensitive units have been described in rabbits by *Frankenhauser* (1949) in the toad and cat by *Maruhashi Mitsuuchi* and *Tasaki* (1952) in the cat by *Zotterman* (1939) *Witt* and *Hensel* (1959), *Hunt* and *McIntyre* (1960 a) *Wall* (1960) *Iggo* (1963 a) and *Tapper* (1964) in cats and monkeys by *Werner* and *Mountcastle* (1965) in monkeys and baboons by *Iggo* (1963 b) and in man by *Hensel* and *Boman* (1961)

A general property of the slowly adapting mechano sensitive units (Type I *Iggo* 1966) of the adult cat is that they respond to a maintained mechanical stimulus with an initially rapid discharge at the onset of the stimulus and then after a few seconds the rate of discharge slowly declines but persists as long as the stimulus is maintained The first phase appears as a peak and the latter as a plateau on the frequency curve

As regards the maximal frequency of the initial peak of the discharge demonstrable in adult cats it is not unusual to observe frequencies of about 400 imp/sec or more (*Hunt* and *McIntyre* 1960 *Tapper* 1964) *Iggo* (1963 a) has reported 1100 imp/sec The persistence of the discharge on sustained mechanical deformation of the skin that is the total duration of the discharge has generally been found to equal the length of time for which the stimulus is maintained Few figures are reported but it is clear that the activity always continues for several minutes afterwards (5 min or more *Iggo* 1966)

### 2 *Present observations*

In the present study it was found that touch units in newborn kittens do not behave in the same way as in the adult cats The maximal frequency that could be produced by physiological stimuli during the initial peak was lower than in adult animals No pronounced adaptation plateau followed after the initial peak Instead the discharge frequency of the units usually dropped to zero very quickly Fig 24 shows parts of three records which were obtained (A) in a newborn (B) a 3 weeks old kitten and (C)

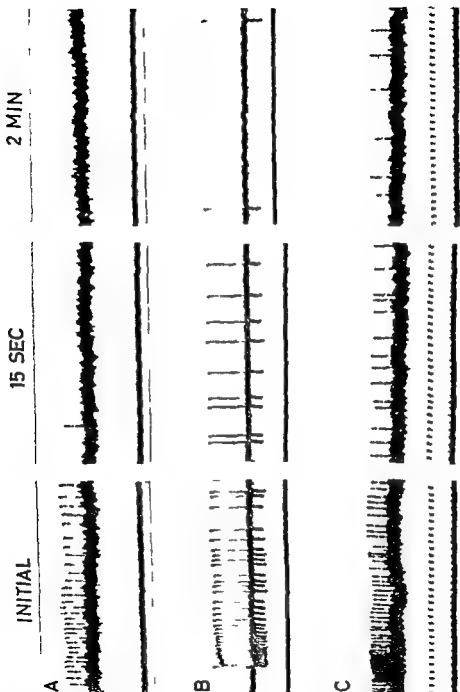


FIG. 24 Recordings from single touch units in (A) newborn (B) 3 weeks old and (C) 6 weeks old kittens. Sustained constant strong mechanical stimulation. The figures indicate time after onset of stimulation. Note low frequency and short duration in A, moderate frequency but long duration in B and high frequency and long duration in C. Note reduced spike amplitude at maximal frequency (Spikes retouched). Time 10 msec.



Fig 25

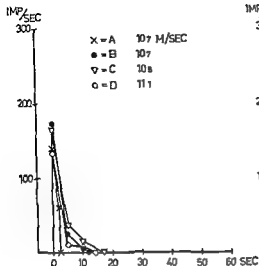


Fig 26

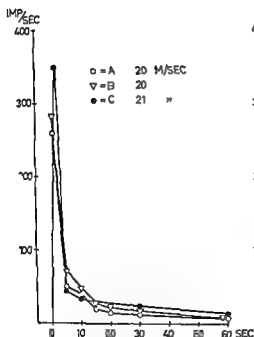
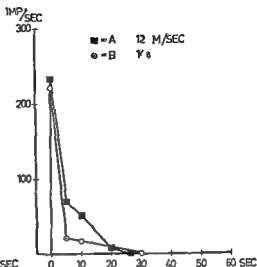


Fig. 27

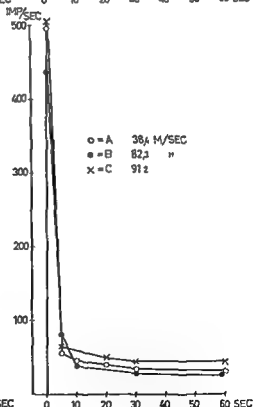


Fig 28

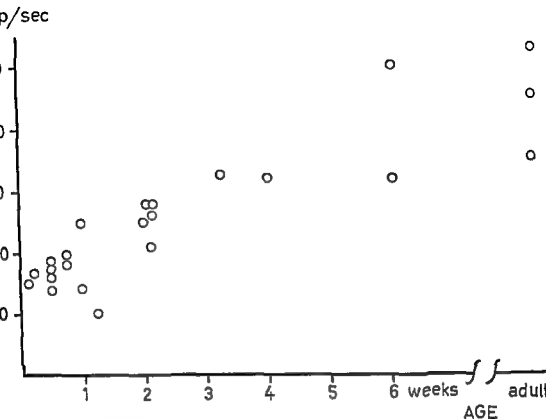
FIGS 25—28 Graphs showing the postnatal changes in the discharge pattern of single touch units in response to the same strong sustained constant pressure. Impulse frequency plotted against time. The unit that gave the highest initial peak frequency in each animal was selected. The receptive fields of the units were situated in the sural skin area. The conduction velocity of the fastest conducting fibres in the suralis is indicated in the figures. Note the increase in peak frequency and discharge duration with

■ 6 weeks old kitten Strong constant stimuli were used As can be seen the initial peak frequency was low in A, higher in B and highest in C After 15 sec the frequency of the unit shown in A dropped to zero whereas the discharge of the other two units continued after 2 min The adaptation frequency was higher in C than in B In the records it can also be seen that the spike amplitude was reduced at the maximal frequency

To further illustrate the postnatal changes of the discharge pattern the frequency of the most efficient (i.e. those giving the highest peak frequency on strong sustained constant pressure by means of the same clip) touch units from a series of animals of different ages have been plotted against time In Fig 25 four frequency curves of touch units from young kittens have been plotted One unit (A) from a kitten with a conduction velocity of 10.7 m/sec in the sural nerve gave an initial peak frequency of 140 imp/sec The frequency dropped to zero after 3 sec In another unit (B) from a kitten with the same conduction velocity the peak was 175 imp/sec and the frequency dropped to zero after 12 sec The third curve (C) shows the response of a unit in a kitten with a conduction velocity of 10.8 m/sec the peak frequency was 170 imp/sec and the duration of the discharge was 17 sec In one case (D) the peak frequency was 170 imp/sec and the duration of the response 14 sec, with a conduction velocity in the sural nerve of 11.1 m/sec

In slightly more mature kittens the units had higher peak frequencies and the frequency dropped to zero after a somewhat longer period of time In Fig 26 a curve (A) is shown from a kitten with a conduction velocity of 12 m/sec in the sural nerve the peak frequency was 235 imp/sec and the duration 26 sec Another unit (B) had a peak frequency of 222 imp/sec which dropped to zero in 30 sec (conduction velocity 14.8 m/sec)

The curves of Fig 27 were taken from kittens with conduction velocities of about 20 m/sec in the sural nerve It can be seen that the type of discharge is changed There is now a plateau of an adapted frequency which is more pronounced than in the early ages The frequency however was low as compared with older kittens and adult cats The discharge persisted for a longer period of time than in kittens with conduction rates below 20 m/sec in the sural nerve A and B illustrate the findings in two kittens with conduction velocities of 20 m/sec in the sural nerve The initial peak frequencies were 260 and 280 imp/sec respectively The units were still firing with frequencies just below 10 imp/sec 1 min after the start of stimulation The unit C was found in a kitten with a conduction velocity of 21 m/sec in the sural nerve It showed a maximal frequency of 350 imp/sec The adapted frequency was 12 imp/sec even 1 min after the start of the stimulation



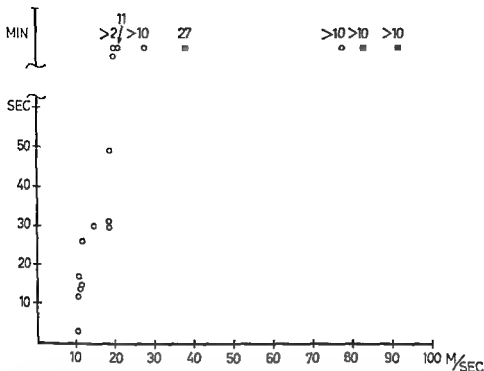


FIG. 30 The duration of the discharge of single touch units in response to the same constant strong sustained pressure plotted against conduction velocity of the fastest conducting fibres in ■ suralis. The receptive fields were localized in the sural skin area. The unit that gave the highest initial peak frequency in each animal was selected.

The minimum interval between 2 spikes obtained by the same stimulation was also determined. It was found that the minimum interval was large (5 msec) in young animals and decreased with increasing age to attain (2 msec or less) adult values at an age of about 6 weeks. This is similar to the results obtained on hair units (Fig. 18).

The duration of the discharge also increased with age. The most immature units adapted rapidly to zero frequency with the persistent constant stimulus and were in this sense phasic. At a certain stage of maturation the plateau-type of the frequency curves appeared. This developmental phenomenon of the discharge duration is illustrated in Fig. 30. The duration of the discharge (the time during which the unit fired on a strong constant maintained stimulus) has been plotted against conduction velocity in the sural nerve. One striking feature of the graph is the abrupt increase of the discharge duration at a conduction velocity of 20 m/sec. From short times of less than 50 sec at conduction velocities up to 19.7 m/sec the duration of

the activity increased to the order of several minutes. This marked increase in the duration occurred at an age of 2—3 weeks, when, as shown in Chapter VI the conduction velocity of the sural nerve reaches 20 m/sec. With a view to determining whether this phenomenon was correlated with the finding of higher maturation of the nerve fibres proximally than distally as described in Chapter V attempts were made to compare the properties of units with receptive fields in the proximal part of the limb with those having their receptive fields in the distal part. However, because of the small number of completely analysed units in each individual animal no conclusions could be drawn.

On the basis of the findings concerning postnatal functional changes described above a somewhat extended analysis was made in an attempt to further elucidate mechanisms limiting the activity of immature touch units.

*Reproducibility of response* The first obvious question is: How reproducible is the response to a constant sustained stimulation? It was found that the frequency curves were fairly reproducible provided that the time interval between stimuli was sufficiently long and that the same stimulus intensity was used.

This is shown in Fig. 31 in a 4 days old kitten. In response to the first sustained constant stimulation the unit adapted to zero in 13.5 sec. The next (identical i.e. with the same clip) stimulus applied after 15 sec of recovery gave a response with a very low initial frequency and a duration of only 0.5 sec. After a recovery period of 2 min the response was still not similar to the initial one. When a recovery period of 10 min was allowed before application of the next stimulus the response was then similar to the initial one i.e. the peak frequency and the duration of the discharge were about the same as in the control response. When a touch unit in a 45 days old kitten was induced to fire for 13.5 sec by the same stimulus and then again after a 15 sec rest period the second stimulus gave approximately the same response as the first. Thus short stimuli can be repeated rather frequently in the more mature animals. The question then arises as to what happens if such a mature unit is made to fire until it has adapted to zero. When the same unit was stimulated until the frequency had dropped to zero (after 27 min) it was found that a 15 minute recovery period was required to obtain a response similar to the initial one. Owing to the great technical difficulties only a small number of complete recovery curves were obtained and thus no definite conclusion can be drawn. It is however quite obvious that the immature sense organs have to be tested very infrequently to allow any conclusions to be drawn with

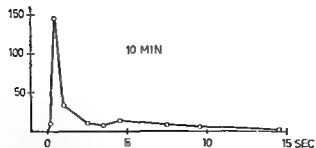
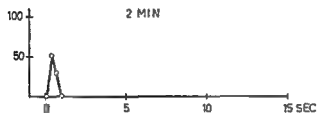
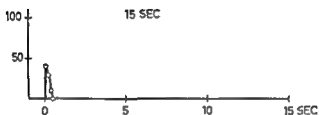
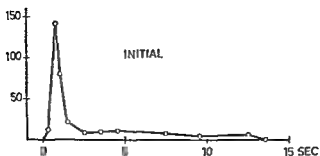


FIG. 31 4 days old kitten The response of a single touch unit to the same constant sustained pressure stimuli repeated after recovery periods of 15 sec 2 min and 10 min Impulse frequency plotted against time

regard to their behaviour under different experimental conditions

When using strong stimuli, the discharge occasionally stopped abruptly and in this way the frequency curve ceased to be smooth. Such units did not usually recover and were discarded because injury could not be excluded

*Strong and weak stimulation* The discharge of adult slowly adapting mechano sensitive units varies with the strength and type of stimulation. The stronger the applied stimulation the higher are the initial peak and adaptation frequencies; the duration of the discharge also increases (cf *Hunt and McIntyre 1960* ■ *Iggo 1963* a *Werner and Mountcastle 1965*)

During postnatal development the relationships between strong and weak stimuli and peak frequency were the same as in adult animals. The duration of the response on the other hand became shorter when the stimulus strength was increased. This is illustrated in Fig 32 in ■ 4 days old kitten. First one strong clip was applied to the receptive field of a touch unit.

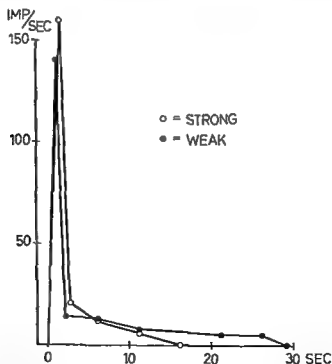


FIG 32 4 days old kitten. The response of a single touch unit to strong (open circles) and weak (filled circles) constant sustained pressure stimulus. Recovery period between the stimulations 15 min. Impulse frequency plotted against time. Note the short duration of the discharge in response to strong stimulation.

After sufficient time for recovery (see above) a second weak clip was likewise applied. It can be seen that strong stimulation gave the highest initial frequency but the shortest duration of the discharge.

Very weak stimuli produced by finger pressure often caused a unit to fire for a considerable period of time. While however use of a clip or a probe lying on the skin can be regarded as giving a constant stimulus level this is not so with finger pressure either directly or via an instrument. Such a non-constant stimulus seemed to give longer duration of the discharge than a constant one in the sense defined here. If the stimulus was intentionally made non-constant by means of alterations of the stimulus strength (rubbing) it could cause a unit to fire for a long period of time if the stimulus was not too strong.

In immature touch units both the maximal firing frequency and duration of the discharge are less than in adult units. With a strong stimulus while the maximal peak frequency is increased the duration of the response is shortened. A weak stimulus has the opposite effect.

*Number of spikes* From the above it is obvious that immature touch units produce overall a smaller total number of spikes in response to mechanical stimulation than adult units. This raises the following question: Is there a difference between the total number of spikes produced by weak and strong stimuli in the same immature unit?

Table X The total number of spikes produced by strong and weak constant stimulation

Age in days	Conductance velocity m/sec	Strength of constant stimulus	Max. peak frequency imp/sec	Duration of discharge sec	Total number of spikes
4	11.6	strong	180	26	582
		weak	160	120	930
5	12.0	strong	200	38	516
		weak	180	130	1065

As can be seen in Table X the total number of spikes produced by a weak constant stimulus was 930 compared with 582 by a strong stimulus. In another experiment 1065 spikes were produced by a weak stimulus and 516 by a strong one. The total number of impulses obtained from weak and strong stimuli thus differ the weak stimulus producing nearly twice as many action potentials as the strong stimulus.

*Electrical stimulation in the skin* It has not been the aim of the present investigation to study the mechanisms responsible for the limitations



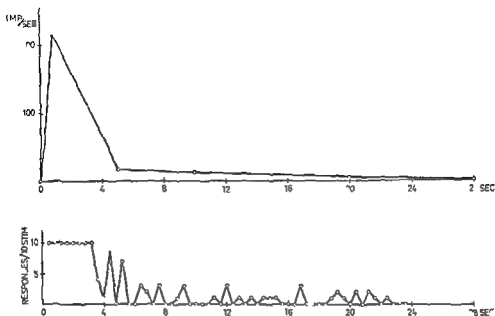


FIG 33 7 days old kitten The response of a single touch unit to a constant strong sustained pressure stimulus (above) and to electrical stimulation in the skin (25 imp/sec) at a stimulus strength just threshold for response (below) Recovery between the two types of stimulation 15 min Impulse frequency plotted against time (above) and responses per 10 stimuli plotted against time (below) Note increase of the threshold to electrical stimulation

of the activity of the sensory units in kittens. Such a study requires other methods. By using some simple experiments however indirect information can be obtained as to which part of the afferent unit may be responsible. The afferent unit consists of a non nervous component in the skin the nerve terminations and the afferent nerve fibre. In order to exclude the mechanical component of the stimulation of the afferent units electrical stimuli were delivered to the skin at the receptive field of the unit. Using short trains of shocks of different frequencies the frequency level at which the one to one stimulus response was broken was determined. It was found that the one to one relation was maintained at higher frequencies in adult cats than in young kittens. This indicates that the capacity of the immature fibre to fire with a high frequency is lower than in adult fibres. This is consistent with the results obtained on hair units.

Electrical stimulation (low frequency) for a long period of time was also tried (Fig 33). After mechanical stimulation and recovery the unit was electrically stimulated at a frequency of 25 imp/sec. When the stimulus strength was supramaximal the units showed a one to one response for

a much longer period of time than when the discharge was induced by mechanical stimulation. When however, the stimulus strength was adjusted to the response inducing threshold value the units showed a one to one response only initially. The number of responses per 10 stimuli then progressively decreased in this case to zero and the latency between stimulus and response increased. When the stimulus strength was increased when the response had reached zero the unit again showed a one to one response. Different durations of the recovery periods were tested with the stimulus strength at just threshold values and unchanged throughout the test series. It was found that stimuli free intervals (recovery periods) of considerable duration (5 min) were required to obtain two similar response series. This shows that the increase of the threshold regardless of its cause lasts for a long time. No definite differences were observed between kittens of different ages. However a tendency for immature units to change these thresholds more quickly than more mature ones was observed.

## E ARTICULAR UNITS

*Single units in dorsal roots* In newborn kittens a few (3) units were identified as articular units localized to the knee joint. These units responded with a discharge of very short duration on mechanical stimulation of the joint capsule and on passive movements. None of these units fired spontaneously for more than a few seconds when the joint was in a resting position.

Some further articular units were isolated in kittens 2—15 days old. The properties of these units did not differ essentially from those described for the newborn kittens.

In one 21 day old kitten an articular unit was found that gave a tonic (> 3 min) discharge when the knee joint was kept in a fixed position. In kittens older than 21 days both this type of tonically discharging articular unit and rapidly adapting ones (duration of discharge about 1 sec) were found. This is quite consistent with Skoglund's (1956) findings that in the adult cat both rapidly and slowly adapting receptors can be found in the joint capsule. The slowly adapting sensory endings fired at a steady frequency for hours if after a period of adaptation from 0.5—1 min they did not reach zero. This means that the above mentioned results i.e. the occurrence of only short duration responses up to an age of 21 days do not allow any conclusions as to whether or not development takes place from phasic to tonic responses. The existence of two types of units and the fact that only a small number of articular units were studied makes it uncertain whether or not the responses of short duration were found by chance in kittens younger than 21 days. In order to study further the

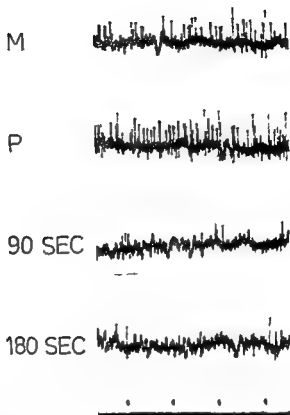


FIG 15 30 days old kitten Recording from the medial knee joint nerve The lower limb is passively moved from  $135^{\circ}$  —  $180^{\circ}$  (maximal extension) and retained there in a fixed position M during movement P just after the fixed position is attained The figures indicate time after P Time 100 msec See text

be produced by movements of the joint or by steady pressure against the capsule and no steady discharge was observed with the joint in a fixed position In a 10 days old kitten responses with somewhat longer duration were obtained both by keeping the joint in a fixed position and by applying constant pressure to the joint capsule In the 30 days old kitten tonic responses which did not adapt to zero for a long period of time were obtained Single unit studies showed that the long duration responses could be obtained in kittens 3 weeks old At this age the fibre composition of the joint nerve (see Chapter V) corresponds to a conduction velocity of about 20 m/sec The results presented here are consistent with unpublished observations of Skoglund (1963)

## Discussion

The method of isolating single active fibres in the dorsal root quite naturally leads to a selection of the material in favour of the biggest fibres which appear to be the most mature ones functionally. When comparisons have been made between units found in kittens of different ages, only the most efficient (with regard to frequency) units of each animal have been chosen. In this way probably the most mature units in each stage have been compared though of course the material may not even be representative for the most mature units. The material can therefore only be evaluated with regard to the developmental trends shown. A strict quantitative analysis of the data might be misleading. As regards the duration of the discharge the recordings from the whole nerves (large population of units) show a fairly good agreement with the single units studied. Any discrepancies found can be attributed to the less satisfactory recording conditions when using undissected nerves or roots.

It might be argued that the limited duration and frequency of the discharge seen in the early developmental stages is due to injury to the units. But the reproducibility of the repeated discharges after a suitable recovery time and the smoothness of the frequency curves makes it unlikely that the limited action should be due to injury. In response to strong stimulation the discharge sometimes stopped abruptly. Such a frequency curve was not smooth and there was usually no recovery. This was interpreted as due to injury and these units were discarded. It is highly improbable however, that the articular units were blocked by injury when the joint was kept in a fixed normal position.

The shapes of the receptive fields of the hair units presented here are essentially similar to those obtained by *Wall* (1960) and *Hunt and McIntyre* (1960 b). The range of the areas of the receptive fields found is consistent with the earlier observations of *Yamamoto et al* (1956), *Puletti* (1959) and *Hunt and McIntyre* (1960 b). The present findings do not however confirm the findings of *Wall* (1960) that the largest area of units sensitive to light brushing of skin or hairs on the proximal hindlimb was only  $18 \times 10$  mm (141 mm<sup>2</sup>) and that the areas changed very little from the foot to the pelvic region. In the present investigation hair units with larger areas than those reported by *Wall* were found on the proximal limb and it was found that the average area increased 3.5 times between the distal part of the foot and the proximal thigh. The discrepancies between *Wall's* findings and those presented here may be due to the different methods used for classification and stimulation of the units.

The areas of the receptive fields in young kittens were smaller than those

in adult cats. This is what might be expected since the total skin area of the hindlimb increases 10 times between birth and the adult stage. This figure is also similar to that found by *Boyd* (1935) for the skin surface increase in man. According to *Szabo* (1958) all hair follicles are already present at birth. The hair follicles must then spread out postnatally concomitantly with the increase in the total skin area. If nervous contact has been established with the hair follicles the receptive areas of each hair unit must also increase. This is supported by the finding that most of the receptive areas of young kittens are smaller than the smallest ones in adult cats. Another possibility is that all hair follicles are not innervated at birth and that such contacts are established during postnatal development.

Although the temperature sensitivity of myelinated mechano sensitive units in the hairy skin of cats is considered to be of minor importance for the sensation of temperature (*Hensel* 1966 a) it is interesting to note that the temperature sensitivity seen in young kittens was essentially the same as in the adult cats. Whether or not it results in sensation cooling evokes an increased activity in the afferent nerves and thus changes in the environment are transmitted centrally in kittens. If cooling gives rise to motor activity the kitten further has a system which enables it to react to a remote stimulus.

From the firing frequencies of afferent units it can be concluded that the receptors subserving different modalities in the skin show the same general traits in their postnatal development. Thus in the newborn stage they have low firing frequencies which gradually increase with increasing age to attain adult values some 40—50 days postnatally. The fibre diameters however continue to increase after this age (Chapter V) but it is interesting to note that the density of mitochondria in the paranodal region attains adult values at about the same time (*Berthold* and *Skoglund* 1967).

All receptors studied adapt relatively rapidly in the newborn stage in response to constant strong stimuli. For those having such properties in the adult stage the development does not of course give rise to any obvious changes. Nor was it possible to investigate their properties other than with regard to firing frequency. The adaptation of the cutaneous receptors developing into slowly adapting units in the real sense on the other hand has been analyzed in considerable detail.

It was then found that the duration of the discharge in response to constant strong stimuli showed an abrupt change at 2—3 weeks postnatally. For the most effective units in the sense defined earlier this change was correlated to the attainment of a conduction velocity of 20 m/sec.

However before this stage of development was reached a strong constant stimulus always gave a short discharge with a high initial frequency and short

duration whereas a constant weak stimulus gave a lower initial frequency but a considerably longer duration of discharge. This is in sharp contrast to the activity obtained from adult receptors where a strong constant stimulus gave a high initial frequency and a high adaptation frequency of long duration with a very large number of spikes. In the immature units on the other hand the weak stimulus produced a greater total number of spikes than the strong stimulus. The articular receptors too gave shorter responses when strong stimuli were used.

The observation of an overall low frequency of immature receptors indicates that the recovery phase of the impulse mechanisms of immature units is not as fast as that of adult units. The finding that the adaptation is faster after a high initial frequency of response points in the same direction with a slow recovery process the overall low ability of the fibre to produce spikes will be more quickly exhausted. This is in good accordance with the observation of an increased relative refractory period found in immature fibres (Skoglund 1960 c) and is further supported by the observation made here that the spike amplitude decreases when the units fire at maximal frequencies in response to mechanical stimulation since this indicates that the spikes are in the relative refractory period of the preceeding spikes (Iggo 1963 a Tapper 1964). Low frequency seems to be characteristic of afferent units in the muscle joint and skin and the same has been found in spontaneously firing diencephalic neurones in young rabbits (Hjvarmen 1966). Thus it appears likely that the low frequency is characteristic not only of some immature peripheral afferent units but also of other immature neurones.

The experiments in which electrical stimulation of the afferent units was used showed that the one-to-one relation between stimulus and response was broken at a lower frequency in the immature units than in the more mature ones. This is in good accordance with the longer absolute refractory period found in immature fibres by Hursh (1939 b) and Skoglund (1960 c). This would also partly explain the low frequencies obtained from immature afferent units on adequate stimulation since their maximal frequency will be limited by that of the fibre.

In the present study an increase of the threshold and the latency was obtained during iterative electrical stimulation of touch units. The effect usually had a duration of about 5 min after interruption of the stimulation. With reservation for the shortcomings of the method the results might indicate that hyperpolarisation of the fibres is induced by iterative stimulation (cf Ekholm and Skoglund 1964 b).

The results of the present analysis of the postnatal functional changes of the exteroceptors in the skin and the proprioceptors in the joint are in good accordance with the observations made by Skoglund (1960 c 1963)

*Weed* (1917) studied reactions induced by skin stimuli in decerebrated kittens. Withdrawal of the leg occurred on pinching the foot or foot pad, the youngest kittens being reported as the most active. In older kittens greater excitation was required but the threshold was still low as compared to that of the adult cats. As regards also other general reactions elicited from the skin *Weed* (1917) emphasized that kittens were all far more responsive to excitations used than are adult decerebrated cats.

*Windle* (1929) found that after decerebration 'pinching the foot pad in kittens resulted in homolateral drawing away of the foot and often the crossed extension followed. A newborn kitten was very irritable to tactile stimuli whereas a 26 days old kitten was 'less irritable and required slightly greater stimuli to produce the same reaction. *Windle* (1930) also investigated behavioral reactions in intact kittens and found that various stimuli cause avoiding responses, the head or the legs tend to move away from the source of the stimulus, and quick reactions that involve many groups of muscles occur when the stimuli are painful.

*Skoglund* (1960 a) found that in decerebrated newborn kittens pinching one hindlimb was followed by withdrawal of that limb and in the contralateral limb it evoked a series of movements of flexion and extension ending in a contralateral flexor reflex. Skin stimuli caused movements in all four legs but the reactions to skin stimuli in the decerebrated preparations were successively reduced during the postnatal development.

The occurrence of rhythmic movements in the limbs in response to skin stimulation has been reported in the prenatal (*Graham Brown* 1915, *Windle* and *Griffin* 1931) and the postnatal period (*Weed* 1917, *Windle* 1929, *Skoglund* 1960 a). Using electromyography *Glebovsky* (1956) recorded activity in knee antagonist muscles of kittens in response to stimulation of the sole of the foot. He claimed that the antagonist activity was both alternating and simultaneous.

In curarized decerebrated or spinal kittens *Malcolm* (1953, 1955) found the first signs of inhibition at 3—5 days of age. He reported that this inhibitory activity came from the skin but no information was given about the type of stimulation used, i.e. whether there was mechanical or electrical stimulation of the skin or electrical stimulation of a skin nerve.

The investigations made (*Weed* 1917, *Windle* 1929, *Skoglund* 1960 a) on the reactions of decerebrate kittens in response to skin stimuli show that such stimuli have a great effect on the motor system whereas the proprioceptive reflexes are less effective (*Skoglund* 1960 a). This dominance of the reflex responses to excitation of cutaneous sense organs is present in spite of the immature behaviour found in these sense organs as shown in Chapter VII.

Thus it remains to consider the second main question posed (Chapter I) of whether the cutaneous sense organs owe their greater influence on motor activity during development to the state of central excitability and organization prevailing at that particular stage of postnatal development

In order to analyze the postnatal changes in the cutaneous reflexes of the kittens the following were studied a) facilitation and inhibition of monosynaptic reflexes by electrical stimulation of a cutaneous nerve and b) the effects that can be induced from the excitatory and inhibitory skin areas for hindlimb muscles using adequate stimulation of the skin as described by Hagbarth (1952)

No information about the excitatory and inhibitory skin areas for hindlimb muscles in animals in the pre or post natal period has been found in the literature. Some of the results presented here were published in a preliminary report (Ekholm 1967)

The question considered in the present work is whether there are differences between young kittens and adult cats with regard to 1) excitability changes of motoneurone pools following a) electrical stimulation (single shocks and iterative stimulation) of a cutaneous nerve and b) mechanical stimulation of the skin 2) the overall response in the muscles induced by mechanical stimulation of the skin and 3) the excitatory and inhibitory skin areas for flexor and extensor muscles

These results can be compared with the state in adult cats since much is known about these mechanisms in the latter (Lloyd 1943 Bernhard 1947, Hagbarth and Naess 1950 Hagbarth 1952 Megirian 1962 Eccles and Lundberg 1959 a b Holmqvist and Lundberg 1961 Lundberg 1962). References to these investigations are made in connection with the experimental results obtained here in kittens

## *Material and Methods*

Successful experiments were performed in 56 kittens ranging in age from 1—90 days

### *1 Experiments with electrical stimulation of n. suralis*

These experiments were performed on animals anaesthetized with intra peritoneal pentobarbital (Nembutal®) the anaesthesia being maintained at as light a level as possible. Generally the dose was 25—35 mg/kg body weight. Lumbosacral laminectomy was performed and the ventral roots L7 and S1 were dissected free and cut near their exits from the dural sac. In the



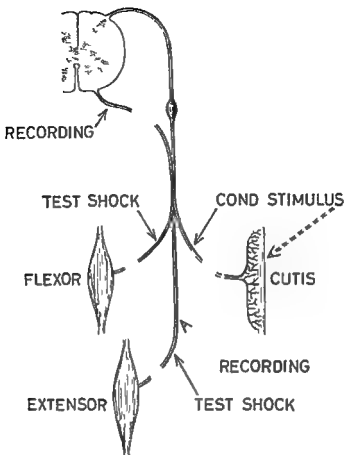


FIG 36 Diagram showing the experimental arrangement when the monosynaptic method was used

popliteal fossa n suralis was cut and dissected free proximally as far as possible One extensor and one flexor muscle nerve were chosen

1) The nerve to the gastrocnemius muscles (both medial and lateral which also includes fibres to the soleus muscle) and 2) the nerve to the biceps semitendinosus muscle These nerves were cut as far distally as possible and dissected free proximally All other hindlimb nerves which were not being studied were cut

For the description of temperature control and protection of exposed tissue see Chapter III and of fixation of the animals Chapter VII

The experimental arrangements are illustrated in Fig 36 The test stimulus was applied either to the flexor or the extensor muscle nerves The conditioning stimulus was applied to the ipsilateral n suralis and the

recording was made from the ventral root (L7 or S1) The conditioning single shocks preceded the test shocks by 1—700 msec

*Stimulation* The conditioning stimulus on n suralis was a square wave of 0.3 msec duration which could be delivered either singly or as a tetanus The stimulus strength was usually supramaximal, this was checked by recording directly from the nerve The test shocks were also square waves of 0.3 msec duration applied to one of the muscle nerves the stimulus strength was usually supramaximal but was sometimes submaximal for the monosynaptic reflex The submaximal stimuli gave very unstable test potentials Two stimulators were used The shocks were triggered by delay circuits To minimize the risk of cross stimulation the shocks were delivered through one to one transformers The sweep intervals were regular and varied between 1 and 3.5 sec the longest intervals being used for the youngest kittens Ag-AgCl hooks were used as stimulating electrodes the interpolar distance was generally 2—3 mm For details about the method of recording see Chapter III

In the experiments with iterative stimulation of n suralis the test reflexes were elicited at regular intervals (usually 2—3 sec in the small kittens and somewhat less in the older ones) and the tetanisation was initiated and interrupted by hand without change of the sweep intervals Thus the first observation of the effect of the tetanisation on the monosynaptic potential was made on the first sweep after the start of the tetanisation The period from the start of the tetanisation to the following first sweep varied within the limits of the sweep intervals used and the events during this early period were not studied in these experiments

## *II Experiments with mechanical stimulation of the skin*

A tracheal cannula was inserted under ether anaesthesia and both carotid arteries were ligated The animals were decerebrated by a section through the intercollicular region The section was made in such a way that the ant sup cerebellar artery was not damaged (see Skoglund 1960 a) One series of kittens were in addition to decerebration spinalized by a transection of the spinal cord at the level Th12 One hour was allowed for the ether effect to disappear

*A Monosynaptic test reflexes* The monosynaptic test reflexes recorded in this series of experiments were elicited from 1) the nerve to the medial and lateral gastrocnemius muscles which also contains fibres to the soleus muscle (as ankle extensor) and 2) the nerve to the anterior tibial muscle or the deep peroneal nerve (as ankle flexor) These muscle nerves were cut near the muscles and dissected free proximally in the popliteal fossa The nerves

to the hamstring muscles were cut. The tibial nerve was usually cut but since it contains cutaneous afferents from the foot and toes it was sometimes left uncut when the effects of cutaneous stimulation applied to the distal parts of the *planta pedis* and digits were being studied.

It was technically very difficult to fix the young kittens satisfactorily when experiments of this type were performed in unanaesthetized decerebrate and/or spinal kittens. When the skin was stimulated mass reflexes in the whole kitten were often evoked. These movements were strong enough to damage any tissue which had been used for fixation. Because of incomplete calcification in the young animals no suitable osseous structures were available for fixation. Denervation could not be made so complete as to exclude all movements, as for example in the axial muscles of the trunk. Curarized animals were not used, the main reason being that possible central effects of this drug in young kittens are not known. However, out of the large number of animals in which these experiments were attempted some were successful and the results will be reported here. Fixation was performed in two ways. Rigid fixation was tried, where the animal was nailed to a cork support. A more flexible type of fixation was also tried to avoid damage to the tissue during mass reflexes. Whether one of the methods was better than the other was never established, both are rather unsatisfactory.

The test potentials were usually recorded from a ventral root (Fig. 36) but in order to obviate some of the fixation difficulties a somewhat modified experiment was performed. The monosynaptic potential was recorded from the same nerve to which the test shocks were applied (Fig. 36) (see Wilson 1962). The stimulating electrodes were situated more distally on the nerve than the recording electrode. This reduced the fixation problem because small movements in parts of the body other than the hindlimb under study did not essentially interfere with the recording. The hindlimb being studied on the other hand was rigidly fixed by nailing it to a cork support.

For further details of the method see also Chapters III and VII. Mechanical stimulation of the skin was performed by pinching with the fingers or forceps or by touching the skin with a probe or a finger (see Chapter VII). The quality of the stimulus varied from a light touch to stimuli which were nociceptive in character.

The sweep intervals were 2.5–3.5 sec in the young kittens which was usually enough to get a fairly stable monosynaptic reflex although the potentials were not maximal if elicited so often (Skoglund 1960 d). In the older kittens somewhat shorter sweep intervals could be used. First a series of fairly stable control test reflexes was established then with the same

sweep conditions the skin was mechanically stimulated for some time and then a new series of controls was recorded

■ *EMG recordings* Reflex responses were recorded electromyographically (EMG) by coaxial electrodes inserted into the muscles through the skin. The position of the tip was controlled by palpation and by the movements of the electrode induced by passive movements of the muscles. Four muscle groups were chosen for EMG recordings: 1) Triceps surae as an ankle extensor (gastrocnemius med and lat soleus and plantaris); 2) anterior tibial muscle (and other pretibial flexor muscles) as an ankle flexor; 3) biceps semitendinosus as a knee flexor; 4) quadriceps as a knee extensor.

Kittens were fixed in the prone position by nails passing through the bones of the limb into a cork support. To check whether any nociceptive stimuli from the nails themselves essentially influenced the reflex pattern, some animals were mounted by tape which should not be expected to cause any painful stimuli. No differences were seen in the experimental results. The results reported have been obtained only from kittens which were in a good condition after the operation.

The activity recorded from the muscles was fed through an amplification system to a loudspeaker and to an oscilloscope which could be photographed (see Chapter III). In order to exclude that the recorded activity arose from adjacent muscles and not from the active muscle into which the electrode was inserted, the presence of spike potentials in the appropriate muscle was always checked. When mapping the excitatory and inhibitory areas, attention was paid only to the response that followed immediately after the stimulus. Usually only one channel was available for recording, but sometimes two EMG recordings could be made simultaneously. The occurrence of inhibition was tested against a background activity set up by stimulation of an excitatory area. The tail was usually used for the excitation of the extensors and the foot pad for the excitation of the flexors (Weed 1917, Skoglund 1960 a).

## Results

### A THE EFFECT OF SINGLE SHOCK STIMULATION OF A CUTANEOUS NERVE ON IPSILATERAL MONOSYNAPTIC TEST REFLEXES

#### 1 Earlier observations on adult cats

Following a conditioning shock to a suralis there is generally an early inhibition and a late facilitation of the ankle extensor motoneurons in adult cats (Bernhard 1947, Hagbarth and Naess 1950, Hagbarth 1952).

Administration of Dial® eliminates the late facilitation and prolongs the early inhibition (Hagbarth and Naess 1950)

An afferent volley from a cutaneous nerve generally causes an early facilitation of flexor motoneurons (Lloyd 1943, Hagbarth and Naess 1950, Hagbarth 1952) and often a late inhibition (Hagbarth 1952) This facilitation was not affected by administration of Dial® (Hagbarth and Naess 1950)

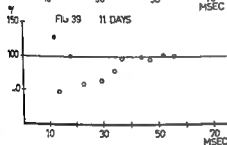
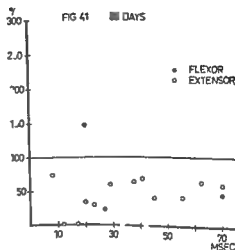
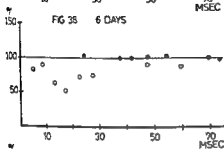
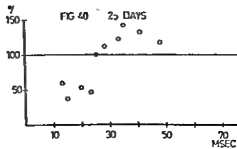
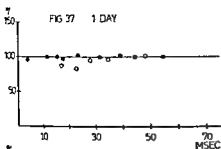
## 2 Present observations

Monosynaptic reflexes from extensor and flexor muscle nerves were recorded from a ventral root in newborn kittens (Skoglund 1960 b) A typical finding from a newborn kitten is shown in Fig 37 which illustrates graphically the amplitude change of the test potentials following a single shock applied to a suralis Each value in the graph (and the following similar graphs) is the mean value of at least 5 and usually 10 consecutive monosynaptic potentials The control values are also the mean of an equal number of observations Supramaximal test stimuli have been used in all the experiments illustrated A very small (105 %) facilitation (control = 100 %) of the flexor test reflex was obtained after 8 msec but this value was within the range of the controls The extensor test potential showed a depression to 74 % of the control 6 msec after the conditioning stimulus and had returned to the control range after 35 msec

In kittens 5—6 days old (Fig 38) a facilitation of the flexor test response with a maximum of 120—135 % was obtained 10—15 msec after the conditioning stimulus In two cases out of five this changed later into a depression beginning 25 msec after the conditioning stimulus The extensor test response changed to an early depression sinking maximally to 53—60 % of the control value 13—18 msec after the conditioning stimulus The amplitude of the test response had returned to the control level after 70 msec in one case out of five in the others it remained depressed for longer periods up to 120 msec

The facilitation of the flexor response in an 11 days old kitten (Fig 39) was 150 % and changed into a late depression The extensor test reflexes showed a depression with a maximum lowering to 42 % of the control about 10 msec after the conditioning stimulus

In Fig 40 the results from a 25 days old kitten are shown The early depression of the extensor test response can be seen The amplitude of the test potential decreased to 37 % of the control level with a minimum after 15 msec After 25 msec the reflex amplitude was back to 100 % and there was then a late facilitation amounting to 142 % with a maximum 35 msec after the conditioning stimulus This curve seems to have the same general shape with an early depression and a later facilitation as those found



FIGS 37-41 Graphs showing the postnatal development of the excitability changes in the motor nuclei of an extensor (gastrocnemius) and a flexor (biceps semitendinosus) following single supramaximal electrical shocks to *n. suralis*. The conditioned amplitudes of monosynaptic test reflexes recorded in the ventral root are expressed in per cent of the control amplitudes at each instance and plotted as a function of the time interval between conditioning and test stimuli. The ages of the kittens are given in the figures. Anaesthetic: Pentobarbital. For further explanation see text.

by Hagbarth (1952) in adult cats although the size of the amplitude changes was not as great in the kittens.

The results illustrated in Fig. 41 were obtained in a 60 days old kitten. The magnitude of the amplitude changes was greater than in any of the younger kittens. The early depression of the extensor test reflex was complete (0 %) between 12 and 17 msec after the conditioning stimulus. The facilitation of the flexor response was 2-3 times the control value. The late depression of the flexor test reflex was also complete (0 %) 33-42 msec after the conditioning stimulus. A late facilitation of the extensor response was not observed but there was a depression amounting to 59 % lasting about 100 msec.

The results in this series of experiments can be summarized as follows following a single conditioning stimulus applied to the ipsilateral sural nerve in newborn kittens a very slight tendency to early facilitation of the flexor motoneurons was sometimes found. A manifest early facilitation was present in 5—6 days old kittens and increased in amplitude during postnatal development. In a 60 days old kitten the facilitation was of a magnitude similar to that found in adult flexor motoneurons. The late depression of the flexor motoneurons was not observed in newborn kittens but at an age of 5—6 days a depression of relatively small amplitude was found. This amplitude increased with increasing age and attained adult values in 60 days old kittens.

Early inhibition of the extensors was present in newborn kittens and increased its magnitude during the postnatal development to attain adult values at the 60 day stage. A late facilitation of the extensor motoneurons was found in a 25 days old kitten.

## B THE EFFECT OF ITERATIVE STIMULATION OF A CUTANEOUS NERVE ON MONOSYNAPTIC TEST REFLEXES IN KITTENS

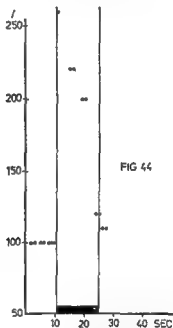
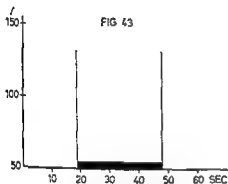
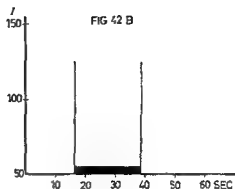
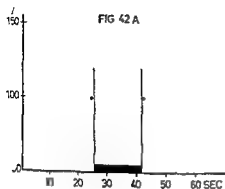
### 1 *Earlier observations on adult cats*

Iterative stimulation of a suralis causes a prolonged facilitation of both ankle flexors and extensors which can be eliminated by Dial® anaesthesia (Hagbarth and Naess 1950).

### 2 *Present observations*

In the experiments made in young kittens although there was sometimes a slight change the amplitude of the monosynaptic test reflexes during iterative stimulation of the ipsilateral sural nerve usually remained unaltered. Relatively low frequencies (80—90 imp/sec) were used in order to avoid blocking the immature fibres (cf. Chapter VII and Skoglund 1960 e). If a change of the response occurred it was usually a slight increase to about 110 %. The same effect was obtained for extensor as well as for flexor test potentials. The facilitation of the monosynaptic potential often lasted only for the first one or two sweeps (sweep interval 2—3 sec).

In an 11 days old kitten the duration of the facilitation was longer as illustrated in Fig. 42A which shows the amplitude of the monosynaptic test reflex elicited from the nerve to the biceps plotted against time. First 10 control test potentials were plotted against time and then the test potentials during tetanisation of the ipsilateral sural nerve. The figure shows a typical result: a relatively slight initial facilitation that declines to control level.



FIGS 42-44 Graphs showing the postnatal development of the excitability changes of the motor nuclei during iterative stimulation of the ipsilateral n. suralis. The conditioned amplitudes of monosynaptic test reflexes recorded in the ventral root are expressed in per cent of the control amplitudes and plotted against time. A series of control test reflexes is shown before and after the test reflexes elicited during iterative stimulation of n. suralis (indicated by the black area). Fig 42 11 days old kitten. A Flexor (biceps semitendinosus) tetanus frequency 80 imp/sec B Extensor (gastrocnemius) tetanus frequency 83 imp/sec Fig 43 14 days old kitten Flexor (biceps semitendinosus) tetanus frequency 167 imp/sec Fig 44 60 days old kitten Extensor (gastrocnemius) tetanus frequency 182 imp/sec Anaesthetic Pentobarbital. For further explanation see text.



control level at the end of a tetanisation lasting 17.5 sec (tetanus frequency 80 imp/sec). After the tetanisation 10 control observations were made.

In Fig. 42B the extensor test potentials are plotted in the same way from the same kitten. The slight facilitation declined during iterative stimulation (83 imp/sec for 22.5) but lasted in this case throughout the tetanisation.

In 14 days old kitten (Fig. 43) the initial facilitation of the flexor test response during tetanisation was somewhat greater (about 130 %) as compared with the findings in the youngest kittens described above. The facilitation declined to control level during a 30 sec long tetanisation at a frequency of 167 imp/sec. Similar effects were usually obtained on the extensor test reflex but in one experiment there was instead a slight depression.

Fig. 44 illustrates the findings in a similar experiment in a 60 days old kitten. The effect of the extensor test reflex during iterative stimulation (frequency 182 imp/sec) for 15 sec was definitely different from that earlier described in young kittens. The initial facilitation was 2—3 times the controls. It declined slowly as in the youngest kittens but it had not returned to the control level after 15 sec. The magnitude and time course of the effects during the iterative stimulation of the cutaneous nerve in a kitten of this age are of the same type as reported by Hagbarth and Naess (1950) for adult cats.

The results obtained in the investigation of the extensor and flexor monosynaptic test reflexes during tetanisation of the ipsilateral sural nerve show that in the newborn stage there was usually no effect. In some cases however a very slight effect usually facilitation occurred. Later during development the facilitation of extensors and flexors was more pronounced. At the age of 60 days the magnitude of the effect was similar to that in adult cats.

## C THE EFFECT OF MECHANICAL STIMULATION OF DIFFERENT SKIN AREAS ON MONOSYNAPTIC TEST REFLEXES

Monosynaptic test reflexes from the ankle flexors (n. per. prof.) set up and recorded peripherally could sometimes be depressed by mechanical stimulation of the skin over the ipsilateral heel in decerebrate newborn kittens. Generally however there was no effect at all unless the stimulus was so strong that mass reflexes were released. Stimuli applied to the anterior part of the limb did not obviously change the test reflexes. Enhancement of the flexor test potential was never observed. The extensor test potential too was usually unaffected by skin stimuli.

When the mass reflexes were present the monosynaptic test potential

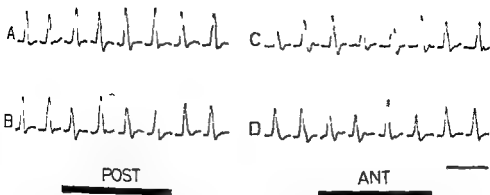


FIG. 45. 8 days old decerebrate kitten. Extensor (gastrocnemius) monosynaptic test reflexes recorded from the ventral root S1. The tracings above the black areas show the test reflexes during pinching of the skin. Control test reflexes are elicited before and after pinching. post = pinching of posterior lower limb (heel) ant = pinching of anterior lower limb. Time one msec. Interval between test potentials 2.5 sec. Note the small magnitude of the amplitude changes, the inconstancy of the type of changes (slight facilitation in A and C, alternating facilitatory and inhibitory effects in B and D) during pinching. Note also the slight facilitation of the extensor induced by pinching the anterior lower limb. For further explanation see text.

was reduced. This is probably due to refractoriness in the motoneurons and thus this method is not very suitable for measuring excitability changes of the ventral horn cells during mass reflexes.

In a 7 days old kitten moderate stimuli which did not release mass reflexes were applied to different parts of the hindlimb skin. Enhancement of the ankle flexor test potential was never seen. The response was unchanged slightly depressed or showed a tendency to alternate between the control and a slightly lower level during mechanical stimulation of the skin. The site of stimulation on the skin appeared to have no effect in determining the type of response.

The alternating type of response was more obvious in an 8 days old kitten in which the extensor test potential was recorded from the S1 ventral root. A test procedure consisting of a pinch of the skin over the heel which is known to be a region of high sensitivity for the excitation of the ankle extensors was repeated 4 times. During mechanical stimulation of the skin over the heel slight facilitation occurred (110—120%) in two trials (Fig. 45A) and in another two trials alternating excitatory and inhibitory effects (B) were recorded. The alternating responses were also of small magnitude. Stimulation of the skin over the gastrocnemius muscle, another potent excitatory area for the ankle extensors, gave also alternating effects.

When the skin of the anterior aspect of the lower limb (which in adult cats is an inhibitory area for this muscle, Hagbarth 1952) was pinched in

the same experiment a slight enhancement of the extensor test reflex (C) occurred in 2 trials. Alternating facilitation and inhibition (D) were found in one trial.

When the area known to have maximal sensitivity for inhibition of the ankle extensor muscles in young kittens, the distal planta pedis was pinched a clear depression of the extensor test reflex occurred.

The experimental data obtained from the young decerebrate kittens may be summarized as follows. The monosynaptic test potentials were found to be relatively unaffected by mechanical skin stimulation. Usually the stimulation did not affect the test potentials, but when it did the changes were rather small. Furthermore the changes recorded during stimulation of a particular region show wide variation. For instance on one occasion alternating changes were obtained and on another slight facilitation was seen. The ankle extensor motoneurons were, however, clearly inhibited from the distal planta pedis and sometimes slightly facilitated from the heel or anterior lower limb in an 8 days old kitten. The ankle flexor motoneurons were usually unaffected but sometimes slightly inhibited from the heel and sometimes they gave alternating responses regardless of the area stimulated.

It can thus be concluded that there is a difference between the young kittens and adult cats with regard to both the magnitude and direction of the changes of the monosynaptic test reflexes in response to mechanical skin stimuli. In adult cats *Hagbarth* (1952) found a marked (up to 2—3 times the control value) facilitation of the gastrocnemius test reflex on adequate stimulation of the posterior lower limb and heel. In the present study with young kittens such stimuli gave only slight facilitation or alternating changes of small magnitude. From the anterior part of the lower limb *Hagbarth* (1952) induced a clear inhibition of the gastrocnemius test reflex in adult cats whereas this area gave a slight facilitation or alternating effects in the young kittens used in the present study.

*Hagbarth* reported that in adult cats the ankle flexor motoneurons were facilitated from the whole limb except for the posterior lower limb and heel. From the latter region inhibition could be induced. This was not observed in the young animals tested in the present study. Enhancement of the flexor test reflex was never seen and only occasionally could slight inhibition be demonstrated from the heel in young kittens.

For comparison with the young kittens the findings from an older kitten (30 days) are shown in Fig. 46. The monosynaptic potential from the ankle extensor was clearly facilitated from the heel and posterior lower limb and inhibited from the anterior lower limb. The ankle flexor test potential on the other hand, was inhibited from the heel and facilitated from the foot and anterior lower limb. Thus in a 30 days old kitten the adult basic

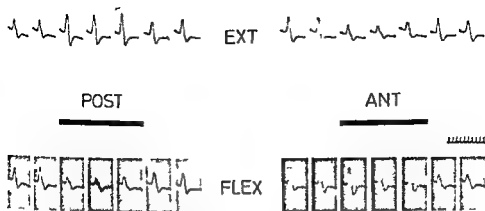


FIG. 46. 30 days old decerebrate kitten. Extensor (gastrocnemius) and flexor (tibialis anterior) monosynaptic reflexes recorded peripherally on the muscle nerves. The tracings above the black areas show the test reflexes during pinching of the skin. post = posterior lower limb (heel) ant = anterior lower limb. Control test reflexes are elicited before and after pinching. Time one msec. Interval between the test reflexes 2 sec. Note the manifest facilitation and inhibition.

pattern was present although the amplitude of the changes of the test reflexes was smaller than that found by *Hagbarth* (1952) in adult cats.

### Comment

According to *Granit* and *Job* (1952) monosynaptic testing gives a measure of the total number of motoneurons firing in response to each synchronous test shock, i.e. the excitability level of those neurons that fire is sufficient for the transmission of a synchronous test shock. *Granit* and *Job* (1952) designated this method a transmission test in distinction to electromyography which they named a discharge test. The two methods were found to be at variance in some respects. In monosynaptic testing, thus facilitation and inhibition mean addition and subtraction respectively of motoneurons which fire in response to a test shock. If the fringe is small or absent, few or no fringe motoneurons are available for addition and therefore in the presence of a conditioning stimulus the control test stimulus has no additional neurons to mobilize and therefore the test response is not changed by conditioning. Reduction of the test shock strength on the other hand may result in a diminished mobilization which may be augmented by more excitable fringe neurons produced by a conditioning shock. Being primarily a fringe test, the monosynaptic test potential technique thus gives no information about the real discharge of the motoneurons. The discharge in the muscle can be recorded, however, by means of EMG. This

method was therefore used in order to get more information about the excitatory and inhibitory skin areas. The EMG is often combined also with myography (Hagbarth 1952, Skoglund 1960 a). For the present study however, it was not considered feasible to dissect free the tendons in the small kittens without damaging the skin innervation and therefore myographic recordings were not attempted.

## D OBSERVATIONS OF REACTIONS TO SKIN STIMULATION IN KITTENS

Typical reactions elicited by skin stimulation in decerebrate kittens were reinvestigated. The results obtained are essentially in accordance with earlier findings. Comment is however made on a few points of interest.

A normally active decerebrate kitten has a high responsiveness for reflex reactions elicited from the skin (Weed 1917, Windle 1929, Skoglund 1960 a). In a young kitten the elicitation of a response very often did not require a painful stimulus but only light touch. In adult cats the evocation of a similar response regularly requires a painful stimulus. The responsiveness decreases during a period of 2—3 weeks postnatally.

The typical response to skin stimulation in a newborn kitten is a reaction which involves many muscle groups and joints (Skoglund 1960 a). Definitely localized responses are very seldom seen in the hindlimbs of newborn kittens but become more frequent with increasing age.

If the distal planta pedis of the hindlimb in a young decerebrated kitten is pinched one or more of the following reactions occur:

- 1) A simple withdrawal of the ipsilateral limb (Weed 1917, Windle 1929, Skoglund 1960 a).

- 2) The initial reaction is an extension which changes into an eventual flexor reflex. The eventual flexor movement can also be preceded by alternating rhythmic flexions and extensions in one or several joints for varying periods of time after the stimulus has ceased (Weed 1917, Skoglund 1960 a).

- 3) In the contralateral limb there may be no response or the initial reaction may be a flexion (Skoglund 1960 a) and extension (Windle 1929) or alternating movements (Skoglund 1960 a). In the event of a reaction the final position may be either flexor (Skoglund 1960 a) or extensor. Final flexion was usual in the youngest kittens and the extension in the older ones.

- 4) Sometimes alternating movements may be present in all four limbs (Weed 1917, Skoglund 1960 a) and occasionally especially with a strong stimulus vigorous longlasting rhythmic activity spreads not only to limb

muscles but also to the trunk and neck (mass reflexes) This occurs more frequently in kittens than in adults

The plantar surface of the hindfoot is the most sensitive area for response but the above reactions can also be elicited from other parts of the limb Stimulation of the skin over the heel often elicited a movement which included a plantar flexion of the foot but was generally followed by a flexor reflex sometimes preceded by alternating movements At an age of about 3 weeks the reactions were adult like

## E EXCITATORY AND INHIBITORY SKIN AREAS FOR EXTENSOR AND FLEXOR MUSCLES AS STUDIED WITH EMG

### 1 *Postnatal changes in the general response in the muscles*

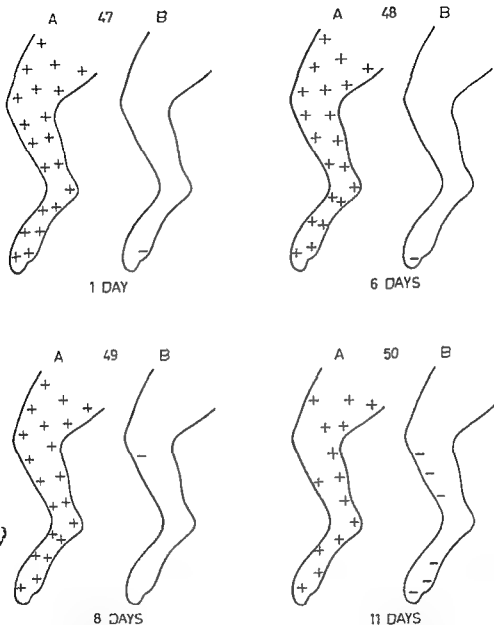
With EMG it is possible to determine which muscles are involved in the movements observed when mechanically stimulating the skin Thus the multi muscular responses involving several joints and the mass reflexes could be recorded in young animals The responsiveness to skin stimuli decreased and the occurrence of alternating responses diminished with increasing age

With increasing age stimulation of one skin locus activity gradually resulted in being limited to more well defined muscle groups Apparently the activity occurred more or less simultaneously in antagonistic muscle groups in response to stimulation of a skin area in newborn kittens (cf Glebovsky 1956) However further studies are necessary before any definite conclusions can be drawn since simultaneous recordings from antagonistic muscles must be made in a faster recording system

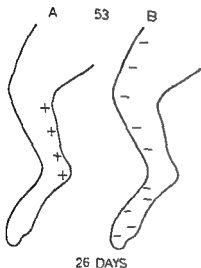
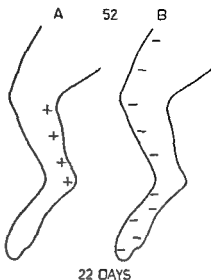
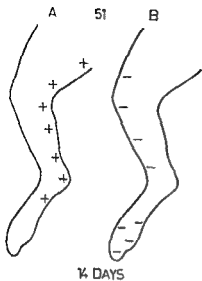
No differences in the response were observed when a short pinch and a sustained constant stimulus were compared in young kittens (cf Chapter VII about adaption of afferent units and earlier in this chapter about excitability changes during iterative stimulation of a cutaneous nerve) But if the stimulus was sustained and increased with time or of an alternating type (i.e. rubbing) or if frequent pinching were used the muscular response could be prolonged

### 2 *Ankle extensors*

Activity in the ankle extensor muscles could be elicited by a moderate stimulus applied to any part of the ipsilateral hindlimb and tail in a newborn kitten The ipsilateral excitatory skin area diminished during the first 2—3 weeks postnatally to an area of the size and localization found in adult cats by Hagbarth (1952) viz the skin area covering the heel and triceps surae muscles on the posterior lower limb Inhibition in response



FIGS 47-53 Diagrams showing the postnatal changes in the excitatory (A) and inhibitory (B) skin areas for the ankle extensor muscles in decerebrate kittens (as studied by EMG) Age = indicated in the figures A plus sign indicates that excitation was induced from this site on the skin (by a pinch) A minus sign indicates that activity set up from an excitatory area was inhibited from this site on the skin Note the postnatal decrease of the excitatory areas and increase of the inhibitory areas For further explanation see text



to noxious stimuli was seldom found in newborn kittens. It was observed however, when stimulating the distal part of the planta pedis that light touch often inhibited the ankle extensor whereas a stronger stimulus caused excitation. The inhibitory area successively spread during the first 2—3 weeks postnatally until it was similar to that found in adult cats by Hagbarth (1952).



The results of a number of experiments are shown schematically in Figs 47—53 and illustrate the postnatal development of the excitatory and inhibitory skin areas for the plantar extensor muscles. In the A figures, which show the excitatory areas, the plus sign means that muscular activity was elicited (if no spontaneous activity was present) or increased (if spontaneous activity was present) from the locus indicated. Only one aspect of the limb is presented in the A figures, the areas were generally symmetrically distributed on the lateral and medial sides of the limb. In the B figures, which show the inhibitory areas, the minus sign indicates that the muscular activity was inhibited from the locus of the skin.

In Fig 47A the response of the ipsilateral limb 10 sec after a skin pinch was shown. The response was of moderate strength throughout (despite many very mild stimuli). A finger active strength of the limb instead immediately.

In a newborn kitten covers the whole activity induced continued for about 15 sec (if reflexes were not elicited). The duration of stimulus was maintained at constant. Inhibition (Fig 47B) could be elicited from the plantar part of the planta pedis only when the foot pad was lightly touched with another source could be inhibited. If the stimulus was increased a little the excitation was

The inhibition in newborn animals (and at 6 and 8 days old as described below) is similar with the inhibition in older kittens. The activity set up by stimulation of an excitatory area could be stopped by a suitable moderate stimulus of an inhibitory area but only very seldom did the muscular activity return spontaneously after removal of the inhibitory stimulus as is a common phenomenon in older kittens and adult cats.

Fig 48 illustrates the results from a 6 days old kitten. Muscular activity could be elicited from any part of the ipsilateral hindlimb skin. In other respects also the findings were similar to those obtained in the newborn kitten. Inhibition was produced by mild stimulation of the digits. Stronger stimuli increased the activity or evoked mass reflexes.

In an 8 days old kitten (Fig 49) muscular activity could be elicited from almost all parts of the hindlimb skin although however, there was a difference in the stimulus intensity required for muscular response between the anterior and posterior aspects of the limb. On the posterior side weak stimuli (touch) gave a response but on the anterior side somewhat stronger stimuli (noxious forceps) were necessary to evoke activity. In this kitten inhibition could only be demonstrated from a small area on the anterior side just distal to the knee joint. Often in kittens of this age, inhibition could also be produced from the distal part of the planta pedis.

In an 11 days old kitten (Fig 50) reflex activity was easily obtained by stimulation of the posterior aspects of the limb. On the anterior side, however only some points gave response if strong pinches were used. Inhibition could easily be produced from the distal planta pedis, the digits and the anterior aspects of the lower limb.

In a 14 days old kitten (Fig 51) the excitatory area was still smaller and covered only the posterior aspects of the limb. The inhibitory area was larger than in the 11 days old kitten, i.e. the anterior sides of both the thigh and lower limb and the dorsal and plantar aspects of the foot and digits.

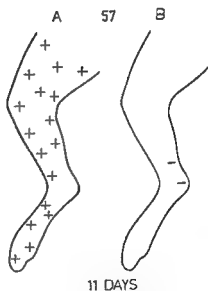
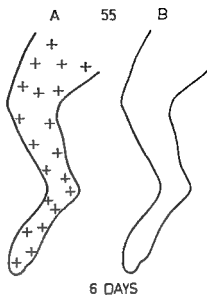
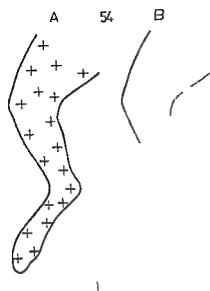
The findings illustrated in Figs 52 and 53 from 22 and 26 days old kittens respectively are essentially similar. The excitatory area was small, being limited to the skin area over the heel and triceps surae muscles on the posterior part of the lower limb. Inhibition could be elicited not only from the distal part of the planta pedis and the digits but also from the anterior aspects of both the thigh and lower limb and the dorsal part of the foot.

The findings described in the two kittens 22 and 26 days old are essentially in accordance with those of Hagbarth (1952) in adult cats.

### 3 Ankle flexors

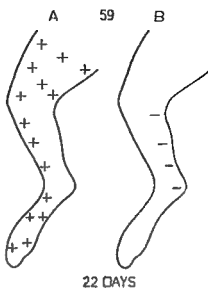
In Figs 54—60 the excitatory and inhibitory skin areas for the ankle flexor are illustrated in the same way as for the extensor in Figs 47—53. Since the trend of the postnatal change of the excitatory and inhibitory skin areas for the ankle flexors is similar to that of the ankle extensors described above, the skin areas attributed to the flexor are described here in a summarized form. The excitatory area covered the whole limb in the newborn and 6-day old kittens (Fig 54A—55A). No inhibition could be demonstrated at this stage (Figs 54B—55B). In the 8 and 11 days old kittens (Fig 56—57) the excitatory area still covered almost the whole limb but a few points on the heel and posterior lower limb gave inhibition. In kittens aged 14 days or older (Fig 58—60) excitation could not be elicited from the posterior lower limb and heel; this area instead gave inhibition. The size and localization of the excitatory and inhibitory areas for the ankle flexors are essentially similar to those found by Hagbarth (1952) in adult cats.

For comparison typical findings in a newborn (A+B) and a 22 days old (C+D) kitten are illustrated in Fig 61. Stimulation of the skin over the heel elicited activity in the ankle extensor at both ages (A, C). The stimulus applied to the anterior lower limb activated also the extensor muscles in the newborn animal (B). This skin area is also situated within the excitatory area of the ankle flexors (see Fig 54). This means that on activation of cutaneous sense organs in a certain locality an excitatory effect can



FIGS. 54-60 Diagrams showing the postnatal changes in the excitatory (A) and inhibitory (B) skin areas for the ankle flexor muscles in decerebrate kittens (as studied by EMG). Other details as in Figs. 47-53.

be induced simultaneously in both the extensors and flexors of the ankle in immature kittens. In the 22 days old kitten (D) on the other hand the extensor activity set up by stimulation of the tail was inhibited by pinching



the anterior lower limb. This shows that an excitatory area in the newborn stage has become an inhibitory area in a more mature stage.

It is thus obvious that the reflex effects from the skin change during postnatal development in such a way that the initially dominating excitatory action on both extensor and flexor muscles elicited from large cutaneous areas is gradually concealed and reverses into an inhibitory action elicitable

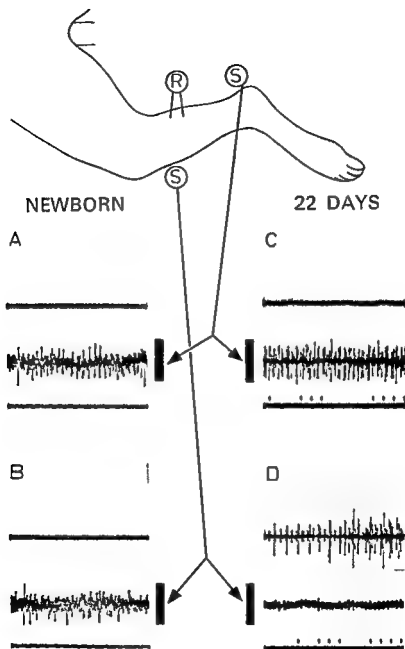


FIG. 61 EMG recording (R) from ankle extensors A and B from a newborn C and D from a 22 days old kitten. Upper tracing: control. Lower tracing: mechanical stimulation (S) of the skin applied to the heel in A and C and to the anterior lower limb in B and D. Time 100 msec. Note that stimulation of the anterior lower limb activated the extensor muscles in the newborn kittens (B) but inhibited the activity in the 22 days old kitten.

from progressively larger skin areas. At the end of this development period the activity has also become reciprocal in the sense that the skin areas which are excitatory for the extensor muscle are inhibitory for the flexor and vice versa.

#### 4 Spinalization

In a number of kittens the experiments were performed both before and after spinalization and in these kittens very few differences were found up to an age of 2—3 weeks. Up to this age no rigidity was obtained in the hindlimbs after decerebration (Malcolm 1955, Skoglund 1960 a). In kittens older than 2—3 weeks the extensor responses were of longer duration and easier to elicit before spinalization. After spinalization the flexor responses were more easily elicited. When the excitatory and inhibitory areas were mapped after the spinalization it was found that as a rule they had not changed to any great extent.

#### 5 Knee extensors and flexors

In order to compare the results obtained in the experiments on ankle muscles with the excitatory and inhibitory areas of the knee muscles the latter were also studied.

In the same newborn kitten the responsiveness of the knee muscles was always lower than that of the ankle muscles. This difference in responsiveness diminished progressively during the development until adult properties were attained at an age of about 2—3 weeks. Thus in this sense the activity of the proximal muscles appears to be more mature than that of the distal ones. This is an interesting observation in view of the finding that muscle spindles in proximal muscles have tonic discharges and gamma innervation earlier than those in distal muscles (Skoglund 1960 c).

The excitatory area of the knee extensors in newborn kittens usually covered the whole limb and gradually diminished up to an age of 14 days. After this age excitation could usually only be obtained from the anterior thigh whereas the rest of the hindlimb skin area caused inhibition. The latter pattern of the areas is similar to that found in adult cats (Hagbarth 1952, Megirian 1962).

In newborn kittens the excitatory knee flexor responses could be evoked from any part of the hindlimb and also from the anterior thigh. Inhibition could not be produced from the anterior thigh. In somewhat older (e.g. 7 days) kittens the responses in the knee flexors could be elicited from almost all parts of the limb. On the anterior thigh however stronger stimuli had to be used for eliciting excitation than on the rest of the hindlimb.

skin areas. In a 22 days old kitten responses could be elicited from all skin areas except the anterior thigh. In kittens of this age pinching the anterior thigh often inhibited the knee flexors. This is consistent with the findings in adult cats (Hagbarth 1952, Megirian 1962) where facilitation of knee flexor motoneurons can be elicited from the whole hindlimb skin except the anterior thigh region from which inhibition is obtained.

It can be concluded that there is a difference in responsiveness between the knee and ankle muscles in newborn kittens: the former are less responsive than the latter (distal) muscles and in this sense the proximal muscles show a more mature behaviour. The trend of the postnatal changes of the excitatory and inhibitory skin areas for the knee muscles is however essentially similar to that for the ankle muscles.

## 6 Responses to stimulation of the contralateral heel

According to Megirian (1962) adequate stimuli applied to the contralateral heel of adult cats generally facilitates the extensor and inhibits the flexor motoneurons. In kittens from 1 to 11 days old activity was induced in both extensors and flexors in response to a pinch of the contralateral heel. From an age of 14 days however the activity generally only appeared in the extensor muscles in response to such a stimulus.

## Discussion

From the analysis of the firing characteristics of immature cutaneous sense organs (Chapter VII) it is obvious that the effectiveness of skin stimuli in producing motor responses in young kittens cannot be due to more efficient firing from these sense organs as compared with muscle receptors. Thus the difference between young and adult animals must be attributed to differences in the central mechanisms of spinal reflexes. However only the properties of myelinated cutaneous afferent units have been analyzed. The myelinated fibres i.e. both group II (alpha and beta) and group III (delta) fibres as well as the unmyelinated fibres contribute to the reflex effects of the ipsilateral limb (Lloyd 1960). In view of the low firing efficiency of the unmyelinated afferent units in the adult state it seems unlikely that in the early stages of development they can compensate for the low firing ability of myelinated afferent units. It cannot be excluded however that functional changes in the reflex effects of cutaneous afferents during development might be due in part to the development of unmyelinated afferents (see below).

Furthermore the local differences in sensitivity for the elicitation of

reflexes between different hindlimb skin areas (the distal planta pedis is the most effective area for the elicitation of the flexor reflex in the newborn and young animal) cannot be explained by a difference in functional maturation of cutaneous sense organs, for two reasons. Firstly the firing properties of the immature cutaneous afferent units depend on the nerve fibre size (Chapter VII) and secondly if anything the nerve fibres of this sensitive reflexogenous area are less developed than the more proximal areas (Chapter V). It is more likely that the explanation lies in the high innervation density of the region (the plantar nerves contain a very large amount of fibres in relation to the size of the surface area which they innervate) and/or the central organisation of these afferents.

The animals used for the experiments with conditioning by electrical stimulation of a cutaneous nerve were anaesthetized with pentobarbital because of the unsatisfactory conditions when such experiments are performed on unanaesthetized decerebrate kittens (see Methods). As shown by Hagbarth and Naess (1952) a similar drug Dial® did not affect the facilitation of flexors and the inhibition of the extensors was somewhat prolonged. The late facilitation of extensors and the facilitation of extensors and flexors in response to iterative stimulation of a cutaneous nerve however were eliminated by this drug. With the light Nembutal® anaesthesia used in the present investigation however both late extensor facilitation and facilitation in response to iterative stimulation of a cutaneous nerve could be demonstrated in older kittens. This indicates that Nembutal® in the dosage used is not as depressant as Dial®. It seems fairly justifiable to compare the results obtained in different animals anaesthetized in the same way although changes in the action of the drug during postnatal development cannot of course be excluded. However it seems highly unlikely that the absent facilitation in newborn kittens can be explained by such an effect since the results were quite in accordance with those obtained in decerebrate kittens. In the experiments where the test potential was recorded peripherally an antidromic effect must be considered. Since Sjöglund (1960 b) showed that such an effect is smaller in newborn kittens than in adult cats and that it lasts for about 100 msec it is not likely that it interferes much with the present results. Wilson (1962) also used the same technique successfully in kittens.

Excitability changes in the motoneurone pools were studied by means of monosynaptic potentials and conditioning by single shocks applied to a skin nerve. A clear facilitation of the flexor motoneurones was not obtained until 5—6 days after birth but there was a small inhibition of the extensors in newborn kittens. To ascertain whether the facilitation effect is absent in the newborn stage because a single conditioning volley in the skin



skin areas In a 22 days old kitten responses could be elicited from all skin areas except the anterior thigh In kittens of this age pinching the anterior thigh often inhibited the knee flexors This is consistent with the findings in adult cats (Hagbarth 1952 Megirian 1962) where facilitation of knee flexor motoneurons can be elicited from the whole hindlimb skin except the anterior thigh region from which inhibition is obtained

It can be concluded that there is a difference in responsiveness between the knee and ankle muscles in newborn kittens the former are less responsive than the latter (distal) muscles and in this sense the proximal muscles show a more mature behaviour The trend of the postnatal changes of the excitatory and inhibitory skin areas for the knee muscles is however essentially similar to that for the ankle muscles

### 6 Responses to stimulation of the contralateral heel

According to Megirian (1962) adequate stimuli applied to the contralateral heel of adult cats generally facilitates the extensor and inhibits the flexor motoneurons In kittens from 1 to 8 days old activity was induced in both extensors and flexors in response to a pinch of the contralateral heel From an age of 14 days, however, the activity generally only appeared in the extensor muscles in response to such a stimulus

## Discussion

From the analysis of the firing characteristics of immature cutaneous sense organs (Chapter VII) it is obvious that the effectiveness of skin stimuli in producing motor responses in young kittens cannot be due to more efficient firing from these sense organs as compared with muscle receptors Thus the difference between young and adult animals must be attributed to differences in the central mechanisms of spinal reflexes However, only the properties of myelinated cutaneous afferent units have been analyzed The myelinated fibres i.e. both group II (alpha and beta) and group III (delta) fibres as well as the unmyelinated fibres contribute to the reflex effects of the ipsilateral limb (Lloyd 1960) In view of the low firing efficiency of the unmyelinated afferent units in the adult state it seems unlikely that in the early stages of development they can compensate for the low firing ability of myelinated afferent units It cannot be excluded however that functional changes in the reflex effects of cutaneous afferents during development might be due in part to the development of unmyelinated afferents (see below)

Furthermore the local differences in sensitivity for the elicitation of

reflexes between different hindlimb skin areas (the distal plantar pedis is the most effective area for the elicitation of the flexor reflex in the newborn and young animal) cannot be explained by a difference in functional maturation of cutaneous sense organs for two reasons. Firstly, the firing properties of the immature cutaneous afferent units depend on the nerve fibre size (Chapter VII) and secondly if anything the nerve fibres of this sensitive flexogenous area are less developed than the more proximal areas (Chapter V). It is more likely that the explanation lies in the high innervation density of the region (the plantar nerves contain a very large amount of fibres in relation to the size of the surface area which they innervate) and/or the central organisation of these afferents.

The animals used for the experiments with conditioning by electrical stimulation of a cutaneous nerve were anaesthetized with pentobarbital because of the unsatisfactory conditions when such experiments are performed on unanaesthetized decerebrate kittens (see Methods). As shown by Hagbarth and Naess (1952) a similar drug Dial® did not affect the facilitation of flexors and the inhibition of the extensors was somewhat prolonged. The late facilitation of extensors and the facilitation of extensors and flexors in response to iterative stimulation of a cutaneous nerve however were eliminated by this drug. With the light Nembutal® anaesthesia used in the present investigation however both late extensor facilitation and facilitation in response to iterative stimulation of a cutaneous nerve could be demonstrated in older kittens. This indicates that Nembutal® in the dosage used is not as depressant as Dial®. It seems fairly justifiable to compare the results obtained in different animals anaesthetized in the same way although changes in the action of the drug during postnatal development cannot, of course, be excluded. However it seems highly unlikely that the absent facilitation in newborn kittens can be explained by such an effect since the results were quite in accordance with those obtained in decerebrate kittens. In the experiments where the test potential was recorded peripherally an antidromic effect must be considered. Since Skoglund (1960 b) showed that such an effect is smaller in newborn kittens than in adult cats and that it lasts for about 100 msec it is not likely that it interferes much with the present results. Wilson (1962) also used the same technique successfully in kittens.

Excitability changes in the motoneurone pools were studied by means of monosynaptic potentials and conditioning by single shocks applied to a skin nerve. A clear facilitation of the flexor motoneurones was not obtained until 5-6 days after birth but there was a small inhibition of the extensors in newborn kittens. To ascertain whether the facilitation effect is absent in the newborn stage because a single conditioning volley in the skin

The reflex patterns in the decerebrate animal were up to two or three weeks after birth essentially unchanged by spinalization, a result also obtained by *Malcolm* (1955) and *Skoglund* (1960 a) This means that the activity of supraspinal structures does not favour the hindlimb extensors in the flexor extensor balance until some time after birth Later, there is a postnatal change of the supraspinal control of the spinal reflexes that must constitute one factor responsible for the excitability changes on the spinal level

However influences on the motoneurons from other sources also change *Skoglund* (1960 a) showed that in decerebrate kittens the tonic stretch reflex was never obtained in the distal hindlimb muscles until some time after birth This means in sharp contrast to the conditions in adult cats, that a prolonged excitation of the motoneurons is not produced by activation of the sense organs in the muscle When a muscle contracts the contraction will only be inhibited by its own Golgi tendon organs causing autogenous inhibition since the muscle spindles lacking gamma support will cease firing in the young kittens (*Skoglund* 1960 c) The discharge from the Golgi tendon organs, though short and phasic may cause facilitation of the antagonist (*Granit* 1950 1955) which may in part be the cause of the alternating movements Recurrent inhibition may also contribute to this phenomenon The excitatory afferent input from the skin is unaffected by such mechanisms therefore it is more effective in activating motor performances in newborn kittens than the proprioceptors of the muscles Thus during postnatal development the source of the most effective excitation of the motoneurons is shifted from exteroceptors in the skin to proprioceptors in the muscles This postnatal change is further evidenced by the findings that proximal muscles are more mature than distal muscles with regard to responsiveness to skin stimuli and as found by *Skoglund* (1960 c) the proximal muscles have tonic discharges from muscle sense organs and gamma innervation earlier in development than distal ones Thus in spite of an increasing afferent inflow from the skin (Chapter VII) the most effective field for inducing motor activity is shifted from the cutaneous sense organs to the muscle receptors and this shift starts before the skin receptors have acquired adult properties but simultaneously with the appearance of tonic discharges and gamma innervation of the muscle spindles Furthermore in spite of the fact that the afferent input set up by electrical stimulation of skin nerves will become less synchronous, because of a wider size distribution among nerve fibres (Chapter V) whereby temporal summation is likely to decrease its effects on the monosynaptic potential will increase Hence the conclusion is drawn that the postnatal reduction of the effectiveness of skin stimuli in producing motor activity and the increased effects on monosynaptic potentials set up by stimulation of skin receptors or afferents cannot be due only if at all to changes in the afferent cutaneous

innervation but rather to changes in the excitability of spinal inter and motoneurons. Even if the synaptic action of skin afferents increases this cannot explain the decrease in the motor effects obtained providing that the inhibitory action does not increase more than the excitatory.

It then remains to discuss the possibility that the postnatal change in the excitatory and inhibitory skin areas might be due to a change in the cutaneous innervation. It might be supposed that the cutaneous sense organs are so organized that in a specific skin area some have excitatory and some inhibitory connections with a specific motoneurone pool and that also in each skin area there is a certain ratio between the excitatory and the inhibitory receptors. Postnatal development leads to an overall reduction of the excitatory effects of skin stimuli and an increase of the inhibitory effects. This is also evidenced by the change in the excitatory and inhibitory skin areas for extensor and flexor motoneurons. The skin areas for excitation decrease and those for inhibition increase. In this process the earlier dominant excitatory effects are depressed but the excitatory connections are still there — the concealed reflexes in the sense of *Sherrington* (*Creed et al* 1932) which can be unmasked under certain experimental conditions as for instance after spinalization (*Hagbarth* 1952, *Job* 1953, *Lundberg* 1962). In this respect the innervation remains unchanged. In view of the magnitude of the excitability changes of inter and motoneurons it is not necessary to assume that a change in the ratio between excitatory and inhibitory receptors in a certain skin area has to occur in order to explain the postnatal change of the skin areas but it cannot of course be excluded. However it should be noticed that the number of myelinated nerve fibres in a cutaneous nerve continues to increase for a long time after the stage of development when the excitatory and inhibitory skin areas present in the adult stage have been established (2—3 weeks). Therefore no great change in the ratio between excitatory and inhibitory afferents takes place after this stage in spite of the considerable change in number of fibres. Consequently it appears less likely that such a change in the ratio between excitatory and inhibitory afferents should have taken place before an age of 2—3 weeks and then stopped. On the other hand a change in the synaptic action in favour of inhibitory afferents might contribute to the change in the effects obtained.

### *Summary*

1) In order to analyze the postnatal changes in cutaneous reflexes of the hindlimb the following experiments were made: a) flexor and extensor monosynaptic test potentials were conditioned by either electrical stimulation (single shocks or tetanus) of an ipsilateral cutaneous nerve in *Littens anaes*

thetized with pentobarbital or by adequate skin stimuli applied to different skin areas of decerebrate kittens and b) EMG was recorded and the excitatory and inhibitory skin areas for extensor and flexor muscles mapped in decerebrate kittens before and after spinalization

2) Following a single conditioning stimulus applied to the ipsilateral sural nerve a manifest facilitation of the flexor test potential was not observed until an age of 5—6 days postnatally but there was a small inhibition of extensors in newborn kittens. The magnitude of the facilitation and inhibition increased progressively up to an age of 60 days postnatally when adult values were attained

3) During tetanisation of the ipsilateral sural nerve in newborn kittens the extensor and flexor monosynaptic potentials were usually unaffected but at later developmental stages a facilitation which attained adult values at an age of 60 days was observed

4) Extensor and flexor test potentials were usually unaffected by mechanical skin stimuli in newborn kittens. In somewhat older kittens small inconstant effects were observed and in 30 days old kittens the effects were adult like

5) By recording the EMG it was found that in the newborn kittens excitatory effects on both flexor and extensor muscles could be elicited from large cutaneous areas whereas the flexors could not be inhibited at all and the extensors only from the distal planta pedis

6) The excitatory effects became gradually concealed as postnatal development proceeded and eventually reversed into inhibitory action elicitable from progressively larger skin areas. The adult pattern of the excitatory and inhibitory skin areas is already present in the 2—3 weeks old kittens. The developmental changes during the first 2—3 weeks furnish an explanation for the existence of concealed reflexes in the adult stage

7) Spinalization did not change the reflex pattern until an age of 2—3 weeks

8) The general responsiveness of the muscles to skin stimuli was high in newborn kittens and decreased with increasing age. In an individual newborn kitten proximal muscles were less responsive to skin stimuli than distal muscles. This difference diminished with increasing age

9) The source of the most effective excitation of the motoneurons shifted postnatally from cutaneous exteroceptors to muscle proprioceptors and the postnatal changes in the cutaneous reflex effects ran in parallel with the appearance of tonic discharges from and gamma innervation of the muscle spindles. When a muscle contracts in the immature kitten it will be auto- genously inhibited since the muscle spindles lacking gamma activation will

cease firing. The cutaneous afferent discharge on the other hand is unaffected by such mechanisms and will be effective in activating the muscles. It is concluded that the postnatal change in the influence of cutaneous afferents both with regard to muscle responsiveness and amplitude alterations of monosynaptic potentials might be explained by a change brought about in the excitability of the interneurons by the increasing influence from muscle sense organs and a gradual appearance of a subliminal fringe in the motoneurone pool although a change in the ratio of excitatory and inhibitory inflows from cutaneous afferents in favour of the inhibitory inflow cannot be excluded.

## CHAPTER IX

# GENERAL DISCUSSION

The aim of the present work was to study the mechanisms which make skin stimuli more effective than activation of muscle receptors in producing motor responses in young kittens. Two main possibilities were considered: 1) whether this is due to a more advanced functional maturation of the cutaneous sense organs as compared with the muscle receptors or 2) whether it is a consequence of immaturity of supraspinal regulation and segmental postural reflexes?

It was shown by morphological fibre analysis that the largest cutaneous myelinated nerve fibres are very small at birth but they gradually increase in diameter subsequently. At an age of 3 months their increase in diameter is still only about 40—50 % of the total increase between birth and the adult stage.

Comparisons between cutaneous nerves supplying different regions of the hindlimb of young kittens indicated that the cutaneous nerves supplying proximal areas are relatively more developed than those supplying distal structures. The skin area of the distal planta pedis — the most effective skin area of the hindlimb for evoking motor responses in newborn kittens — is thus not supplied by nerve fibres that are more highly developed morphologically than those supplying other cutaneous structures of the limb. The postnatal development of nerve fibres supplying the distal planta pedis is quite similar to those supplying the dorsal aspect of the foot, a region that is less effective than the distal planta pedis in evoking motor responses. The diameters of cutaneous nerve fibres increase more slowly than those of muscle nerves (*Skoglund and Romero 1965*). It was found that there is a continuous addition of myelinated nerve fibres in the suralis during about the first 2—3 months postnatally. Thus there are no morphological findings indicating that the cutaneous afferents are more developed than the muscle afferents. Articular afferents were found to develop at the same speed as the cutaneous afferents.

A relationship between conduction velocity and nerve fibre diameter was established, enabling the fibre diameter to be calculated from the conduction

velocity and vice versa. A factor of 4.7 (conduction velocity/fibre diameter) was used since the well known value of 6 (Hursh 1939 a) was shown to be too high, especially for slow conduction velocities. The conduction velocity was found to increase by 0.6 m/sec per day postnatally. This value is less than that of muscle afferents i.e. 1 m/sec per day (Skoglund 1960 b) which is in accordance with the morphological findings that cutaneous afferents develop more slowly than muscle afferents.

By recording from peripheral cutaneous afferent units it was found that hair units and touch units could only be brought to fire at low frequencies in response to adequate stimulation in newborn kittens and that the ability to respond with higher frequencies increased during a period of about 40—50 days postnatally. Furthermore the duration of the discharge of touch units in response to a sustained stimulus was very short in newborn kittens but increased rather abruptly to adult values at the stage when the conduction velocity in the cutaneous nerves reached 20 m/sec. This relationship between change of discharge duration and conduction velocity was also found in articular afferent units. Skoglund (1960 c) also found a similar relationship in muscle sense organs. It therefore seems probable that it is a phenomenon of general occurrence in the postnatal development of peripheral afferent units and that the functional change is associated with changes in the nerve fibre properties rather than in receptor structure, since the same relationship between discharge and nerve fibre properties has been found in three different structures viz muscle joint and skin. Muscle afferents attain a conduction velocity of 20 m/sec earlier during postnatal development than cutaneous afferents (see above) which is the reason that the change from phasic to tonic discharges must occur earlier in muscle sense organs than in cutaneous sense organs. It is therefore evident that a more advanced functional maturation of myelinated cutaneous peripheral afferent units as compared with those of muscle sense organs could not be the explanation for the effectiveness of cutaneous stimuli in producing motor responses. On this basis the spinal reflex organization of the newborn kitten becomes of interest.

The postnatal changes in cutaneous reflex effects had already begun before the cutaneous sense organs had reached adult functional maturation and earlier than supraspinal influences could be shown to exert their action on the spinal reflex as evidenced by the comparisons of the findings obtained before and after acute spinalization. The appearances of tonic discharges from and gamma innervation of the muscle proprioceptors, on the other hand occur at the same time as the changes in the cutaneous reflex effects. When a muscle contracts in the immature kitten it will be



autogenously inhibited, since the muscle spindles lacking gamma activation will cease firing but the cutaneous afferent discharge which is unaffected by such mechanisms will be effective in activating the muscles. Thus the source of the most effective excitation of the motoneurons is shifted postnatally from cutaneous exteroceptors to muscle proprioceptors. Furthermore the postnatal change in the effect of cutaneous afferents both with regard to muscle responsiveness and amplitude alterations of monosynaptic potentials could be explained by the gradual appearance of a subliminal motoneurone fringe. A change in the ratio of excitatory and inhibitory inflows from myelinated as well as unmyelinated afferents in favour of the inhibitory inflows also cannot be excluded though it appears unlikely. Postnatally the reflex effects evoked from the skin show a progressive decrease of excitatory action with an increase of inhibitory activity. In this process the dominant excitatory influences from the skin in the early developmental stage become concealed with increasing age and thus postnatal development provides an explanation for the phenomenon of concealed reflexes in adult cats.

It might be expected that in general newborn kittens would have a rather poor capacity to adapt themselves to the environment due to deficient function in several systems e.g. the visual (the eyes are closed) and the proprioceptive systems. However, it is evident that the deficient proprioceptive mechanisms in the muscles are partly compensated for by the effectiveness of the cutaneous sense organs in evoking motor performances such as avoiding reactions and locomotion of the crawling type by means of alternating movements. Thus for the period of time during development before the muscles acquire gamma innervation the firing of the motoneurons is much influenced by afferent inflow from sources other than muscle receptors. This situation is in some ways similar to that existing for those muscle groups which lack muscle spindles in the adult stage e.g. the afferent inflow to hypoglossal motoneurons comes from submucosal tongue sense organs connected to the lingual or glossopharyngeal nerve in cats (Blom 1960) and a similar situation has been reported in laryngeal muscles (Mårtensson 1964).

However the lack of inhibitory effects may lead to excessive motor activity in response to a persistent skin stimulus. This might be counteracted in part by short lasting discharges of the immature cutaneous sense organs. Remote as well as contact thermal stimuli may play a role in the activity of newborn kittens e.g. a decrease of the temperature gives rise to activity in the afferent cutaneous nerves and if the stimulus is sufficient it may evoke motor activity. Such an ability of the afferent units might be utilized for example for locomotion and orientation in relation to the

mother cat which emits radiant heat. The paucity of inhibitory mechanisms seems to be good neuronal economy in an immature organism which has to react to changes in the environment in spite of poorly developed metabolic abilities i.e. in the sense that in spite of the highly limited afferent impulse production of immature cutaneous sense organs (in response to a skin stimulus) the stimulus gives rise to considerable motor activity.

Cutaneous reflexes are a valuable tool in clinical diagnostic work e.g. the Babinsky sign. The mechanisms involved in cutaneous reflexes in man have been studied with neurophysiological methods (*Hagbarth and Kugelberg 1958, Kugelberg and Hagbarth 1958, Eklund, Grimby and Kugelberg 1959, Hagbarth 1960, Kugelberg, Eklund and Grimby 1960, Hagbarth and Finer 1963, Grimby 1963*). Cutaneous reflex mechanisms in babies, however, have been studied relatively less with such methods. The H reflex on the other hand has been studied by *Blom, Hagbarth and Skooglund (1964)* and appears to be functionally more mature than in newborn kittens with regard to posttetanic potentiation. With regard to the nerve fibre sizes of newborn babies (*Nystrom and Skooglund 1965*) those of the cutaneous nerves appear to correspond roughly to the developmental stage at which the discharge duration of cutaneous touch units attains adult values in kittens. Though great caution must be exercised in applying findings in cats to the situation in man the present experimental findings may be useful with respect to the immature sensorimotor arc in newborn infants.

## GENERAL SUMMARY

The work reported here is an investigation of the postnatal functional changes in the cutaneous and articular sense organs, the growth in diameter of their connecting nerve fibres and the postnatal changes in the cutaneous reflex patterns in kittens.

In the first chapter the main theme of the study is presented. It is known that in contrast to the small effects resulting from activation of muscle receptors skin stimuli are very potent in evoking motor responses in young kittens. Two possible explanations for this phenomenon are that it is due to 1) more advanced functional maturation of the cutaneous sense organs as compared with muscle receptors or 2) a lack of supraspinal regulation and absence of segmental postural reflexes.

The anatomy of the cutaneous nerves in the hindlimb and the nomenclature used are presented in Chapter II. In Chapter III the segmental origin of different hindlimb cutaneous nerves was studied by electrical stimulation and recording. The composition of the segmental origin varies considerably between animals and the constituent fibres of a nerve do not always pass through consecutive rootlets. It was found, however, that some fibres of a particular nerve often entered a certain rootlet. The latter finding is useful when single dorsal root fibres contributing to a certain nerve must be identified. Some observations on the distribution of hindlimb dermatomes are reported in Chapter IV. In contrast to an earlier investigation it was found that the S1 dermatome usually reaches distally down to the middle of the foot.

In Chapter V the postnatal development of the fibre compositions of cutaneous and articular nerves were studied in osmium stained sectioned material. The size of the myelinated nerve fibres of cutaneous nerves was very small in the youngest kittens. The diameter range of the nerve fibres increased during postnatal development and the increase of the largest fibres at an age of 90 days was only about 40—50 % of the total increase between birth and the adult stage. The development of the articular nerve fibres (knee joint) was similar to that of the cutaneous nerves of the hindlimb. The diameter increase of the largest cutaneous and articular nerve fibres was

slower than that of muscle nerves. Comparisons between different cutaneous nerves gave results consistent with a cephalocaudal and proximo-distal development.

Chapter VI deals with postnatal changes of the conduction velocity in the sural nerve. The conduction velocity increased by approximately 0.6 m/sec per day or 1 mm/sec per one mm increase of the femur length. The conduction time for a stretch of nerve equal to the femur length decreased postnatally. The developmental stage at which a nerve attains a maximal conduction velocity of 20 m/sec is of particular interest with regard to the contemporaneous changes in the physiological properties of the sensory units. The largest fibres of such a nerve were calculated to have a diameter of  $4.3 \mu$ . A factor of 4.7 was used for this calculation because Hursh's factor of 6 was shown to be too high, especially for slow conduction velocities.

In Chapter VII the postnatal changes in the properties of the discharge from cutaneous and articular peripheral afferent units were investigated. By means of single unit recordings in the dorsal root it was found that hair units and touch units could only be brought to fire at low frequencies in response to mechanical stimulation of the skin in newborn kittens and that the ability to respond with higher frequencies increased progressively up to an age of about six weeks. The duration of the discharge of touch units in response to a sustained constant mechanical stimulus is very short in newborn kittens and the duration abruptly increases to adult values at the stage when the conduction velocity of the cutaneous nerve reaches 20 m/sec. The same relationship between change of discharge duration and fibre size applies for articular afferent units and since a similar relation has been described in muscle sense organs it appears to be a general phenomenon in the development of peripheral afferent units. Electrical stimulation of the skin in the receptive fields of hair and touch units indicated that the ability of the immature nerve fibres to fire with a high frequency is lower than that of adult fibres. In contrast to the property of touch units in adult cats immature units gave discharges of shorter duration and with a small total number of spikes in response to strong stimulation than in response to weak stimulation. The articular units behaved similarly in this respect.

In Chapter VIII the postnatal changes in the cutaneous reflexes were analyzed. By recording monosynaptic test potentials and conditioning stimulation of 1) a cutaneous nerve or 2) the cutaneous sense organs (mechanically) it was found that a manifest facilitation was not obtained until an age of 5—6 days postnatally. Subsequently facilitation and inhibition gradually increased in magnitude and in 60 days old kittens the findings were adult like.

By means of EMG recordings it was found that in the newborn kitten excitatory effects in both flexor and extensor muscles could be elicited from large cutaneous areas. No skin area was found which inhibited the flexor activity induced from an excitatory area. Extensor activity could only be inhibited from the distal planta pedis. The excitatory effects from a certain skin area gradually became concealed during postnatal development and reversed into inhibitory action elicitable from progressively larger skin areas. The adult pattern of the excitatory and inhibitory skin areas is present in 2—3 weeks old kittens. The developmental changes thus give an explanation for the concealed reflex present in the adult stage. Spinalization did not change the reflex pattern until an age of 2—3 weeks postnatally indicating that there is a postnatal change in supraspinal influences on the spinal reflexes that may constitute one factor responsible for the excitability changes at the spinal level. The source of the most effective excitation of the motoneurons is shifted postnatally from cutaneous exteroceptors to muscle proprioceptors and the postnatal changes in the cutaneous reflex effects run in parallel with the appearance of tonic discharges from and gamma innervation of the muscle spindles. When a muscle contracts in the immature kitten it will be autogenously inhibited since the muscle spindles lacking gamma activation will cease firing. The cutaneous afferent discharge on the other hand is unaffected by such mechanisms and will be effective in activating the muscles. It is concluded that the postnatal change in the influence of cutaneous afferents both with regard to muscle responsiveness and amplitude alterations of monosynaptic potentials may be explained by a gradual change of the excitability of the interneurons and a gradual appearance of a subliminal fringe in the motoneurone pool although a change in the ratio of excitatory and inhibitory inflows from cutaneous afferents in favour of the inhibitory inflow cannot be excluded.

Chapter IX is a general discussion of some of the results reported in the preceding chapters. As regards the main questions posed it can be concluded that a greater efficiency in the discharge of the cutaneous sense organs as compared with muscle sense organs is not the explanation for the effectiveness of cutaneous stimuli in producing motor responses in young kittens which must therefore be due to the functional organization of the spinal cord. It is probable that the postnatal changes in the cutaneous reflex patterns also can be attributed to functional changes in the spinal cord although a change in the ratio between excitatory and inhibitory inflows from cutaneous afferents cannot be excluded.

## ACKNOWLEDGMENTS

This investigation was carried out at the Department of Human Anatomy University of Uppsala and the Department of Anatomy Karolinska Institutet Stockholm. I wish to extend my thanks to the Heads of these departments Professors Bror Rexed and Ture Petren for their support and for allowing me to use the facilities of their Institutes.

My thanks are due to Associate Professor Sten Skoglund for introducing me to experimental neurophysiology for suggesting the subject of this study and for his helpful criticism and advice.

I wish to thank the scientific staffs of these Institutes and also Docent K. E. Hagbarth and Docent A. Vallbo for valuable discussions.

I am grateful to Mrs. Sonja Schwieler, Miss Inga Lill Andersson and Miss Annika Åkerlund for technical help with the morphological analysis.

Finally I would like to thank Miss Maj Berghman for her skilful drawing of the illustrations and Miss Annette Wijkman for typing the manuscript.

This work was supported by grants from the Medical Faculties of the University of Uppsala and Karolinska Institutet, the Swedish Society for Medical Research and the Swedish Medical Research Council (Project No B67 12x 136 03A).

Uppsala May 1967

*Jan Ekholm*

## REFERENCES

- Adrian E D and Y Zotterman* The impulses produced by sensory nerve endings Part 2 The response of a single end organ *J Physiol (Lond)* 1926 a 61 151—171
- The impulses produced by sensory nerve endings Part 3 Impulses set up by touch and pressure *J Physiol (Lond)* 1926 b 61 465—483
- Andrew B L and E Dotti* The deployment of sensory nerve endings at the knee joint of the cat *Acta physiol scand* 1953 28 287—296
- Angulo y Gonzalez A W* The prenatal development of behavior in the albino rat *J Comp Neurol* 1932 55 395—442
- Neurological interpretation of fetal behavior The development of the sensory system in albino rat *Anat Rec* 1937 67 suppl 4
- Arutyunyan R S* The development of processes of inhibition and facilitation in a monosynaptic spinal arc in the early post natal period *Doklady Akad Nauk SSSR* 1961 140 260—263 (English translation *Proc Acad Sc USSR Biol Sc Sect* 1962 140 897—899)
- Bernhard C G* Slow cord potentials of opposite sign correlated to reciprocal functions *Acta physiol scand* 1947 14 suppl 47 6 1—25
- Berthold C H and Skoglund* Ultrastructure and histochemistry of the developing node of Ranvier in the hindlimb nerves of the cat *Acta Soc Med Upsal* 1965 70 287—293
- Histochemical and ultrastructural demonstration of mitochondria in the paranodal region of developing feline spinal roots and nerves 1967 72 36—70
- Blom S* Afferent influences on tongue muscle activity *Acta physiol scand* 1960 49 suppl 170
- Blom S A E Hagbarth and S Skoglund* Posttetanic potentiation of the H reflexes in infants *Exper Neurol* 1964 9 198—211
- Boyd E* The growth of the surface area of the human body Minneapolis Minnesota Univ Minnesota Press 1935
- Boyd I A* Nerve impulses from the proprioceptors in the knee joint of the cat *J Physiol (Lond)* 1953 119 8P
- The histological structure of the receptors in the knee joint of the cat correlated with their physiological response *J Physiol (Lond)* 1954 124 476—488
- Boyd I A and T D M Roberts* Proprioceptive discharges from stretch receptors in the knee joint of the cat *J Physiol (Lond)* 1953 122 38—58

- Brown A G and A Iggo* Hair follicle receptors with myelinated afferent nerve fibres *J Physiol (Lond)* 1964 172 33P
- Carmichael L* Origin and prenatal growth of behavior In *A handbook of child psychology* Ed C Murchison Worcester Clark University press 1933 31—159
- An experimental study in the prenatal guinea pig of the origin and development of reflexes and patterns of behavior in relation to the stimulation of specific receptor areas during the period of active fetal life *Genet Psychol Monogr* 1934 a 16 337—491
- The genetic development of the kittens capacity to right itself in the air when falling *J Genet Psychol* 1934 b 44 453—458
- Ontogenetic development In *Handbook of experimental psychology* Ed S S Stevens New York Wiley 1951 281—303
- The onset and early development of behavior In *Manual of child psychology* Ed L Carmichael New York Wiley 1954 60—185
- Cedergren E* Ultrastructure of motor end plate and sarcoplasmic components of mouse skeletal muscle fibre *J Ultrastruct Res* 1959 suppl 1
- Celesia G G* Segmental organization of cortical afferent areas in the cat *J Neurophysiol* 1963 26 119—206
- Coghill G E* Correlated anatomical and physiological studies of the growth of the nervous system of Amphibia I The afferent system of the trunk of *Amblystoma* *J Comp Neurol* 1914 24 161—233
- Correlated anatomical and physiological studies of the growth of the nervous system of Amphibia II The afferent system of the head of *Amblystoma* *J Comp Neurol* 1916 26 247—340
- Correlated anatomical and physiological studies of the growth of the nervous system of Amphibia V The growth of the pattern of the motor mechanism of *Amblystoma punctatum* *J Comp Neurol* 1926 a 40 47—94
- Correlated anatomical and physiological studies of the growth of the nervous system of Amphibia VI The mechanism of integration in *Amblystoma punctatum* *J Comp Neurol* 1926 b 41 95—152
- Correlated anatomical and physiological studies of the growth of the nervous system in Amphibia IX The mechanism of association of *Amblystoma punctatum* *J Comp Neurol* 1930 51 311—375
- Coronios J D* Development of behavior in the fetal cat *Genet Psychol Monogr* 1933 14 283—386
- Creed R S D Denny Brown J C Eccles E G T Liddell and C S Sherring-ton* Reflex activity of the spinal cord Oxford The Clarendon Press 1932
- Eccles R M and A Lundberg* Synaptic actions in motoneurons by afferents which may evoke the flexion reflex *Arch Ital Biol* 1959 a 97 199—221
- Supraspinal control of interneurons mediating spinal reflexes *J Physiol (Lond)* 1959 b 147 565—584
- Ekholm J* Some properties of cutaneous sense organs during postnatal development (preliminary note) *Acta Soc Med Upsal* 1965 70 225—258
- Postnatal development of excitatory and inhibitory skin areas for hindlimb muscles in kittens (preliminary note) *Acta Soc Med Upsal* 1967 72 20—24



- Eklholm J G Eklund and S Skoglund* On the reflex effects from the knee joint of the cat *Acta physiol scand* 1960 50 167—174
- Eklholm J and S Skoglund* Intra articular knee joint temperature variations in response to cooling and heating the skin in the cat *Acta physiol scand* 1960 50 175—185
- Autonomic contributions to myelinated fibres in peripheral nerves *Acta Morphol Neerlandica Scand* 1964 a 6 55—63
- Possible factors influencing the demonstration of post tetanic potentiation of the H reflex as studied in the cat *Exper Neurol* 1964 b 9 183—197
- Eklund G and S Skoglund* On the specificity of the Ruffini like joint receptors *Acta physiol scand* 1960 40 184—191
- Eklund K L Grimbv and E A* Nociceptive reflexes of the human foot. The plantar responses *Acta physiol scand* 1959 47 297—298
- Frankenhauser B* Impulses from cutaneous receptor with slow adaption and low mechanical threshold *Acta physiol scand* 1949 18 68—74
- Gardner E* The distribution and termination of nerves in the knee joint in the cat *J Comp Neurol* 1961 111—32
- Glebovsky I D* Reflexion and decerebrate rigidity in early postnatal ontogeny *Acta Med (in English translation)* 1956 41 908—913
- Graham Brown T* The development of the fundamental activity of the nervous system in progress: a basis of the conditioning of rhythmic activity of the evolution of function of the nervous system *J Biol Sci* 1914 48 18—46
- On the development of the central nervous system of the unborn foetus of the cat with special reference to the question whether progression is a learnt process *J Biol Sci* 1915 49 208—215
- Granit R* *An Introduction to the Study of the EEG* *Clin Neurophysiol* 1950 2 417—424
- *Receptors and Perception* New Haven Yale University Press 1955
- Systems for control of movements *Congr Int Sci Neurol Brussels Ed Acta Med Belg* 1957 1 63—80
- Granit R and C Jol* Electromyographic and monosynaptic definition of reflex excitability during muscle stretch *J Neurophysiol* 1952 15 409—420
- Grimby L* Normal plantar response: integration of flexor and extensor reflex components *J Neurol Neurosurg Psychiat* 1963 26 39—50
- Hagbarth A E* Excitatory and inhibitory skin areas for flexor and extensor motoneurons *Acta physiol scand* 1952 26 suppl 94
- Spinal withdrawal reflexes in the human lower limbs *J Neurol Neurosurg Psychiat* 1960 23 222—227
- Hagbarth A E and B Finer* The plasticity of human withdrawal reflexes to noxious skin stimuli in lower limbs. In *Progress in brain research vol 1 Brain mechanisms* Ed G Moruzzi, A Fessard and H Jasper Amsterdam Elsevier 1963 65—78
- Hagbarth A E and E Kugelberg* Plasticity of the human abdominal skin reflex *Brain* 1958 81 305—318

- Hagbarth A E and K Naess* Reflex effects of tetanic stimulation of different afferent fibre systems in the hindlimb of the cat *Acta physiol scand* 1950 21 336—361
- Harrison H M* Dissection of the cat Saint Louis Mosby company 1962
- Hekmatpanah J* Organization of tactile dermatomes C1 through L4 in cat *J Neurophysiol* 1961 24 129—140
- Hensel H* Classes of receptor units predominantly related to thermal stimuli In Touch heat and pain Ciba foundation symposium 1965 Ed A V S de Reuck and J Knight London Churchill 1966 a 275—290
- *Allgemeine Sinnesphysiologie Hautsinne Geschmack Geruch* Berlin Springer Verlag 1966 b
- Hensel H and K Boman* Afferent impulses in cutaneous sensory nerves in human subjects *J Neurophysiol* 1960 23 564—578
- His W* Die Neuroblasten und deren Entstehung im embryonalen Marke *Abh math physik kl Sachs Akad Wiss* 1889 15 311—372
- Holmqvist B and A Lundberg* Differential supraspinal control of synaptic actions evoked by volleys in the flexion reflex afferents in alpha motoneurons *Acta physiol scand* 1961 54 suppl 186
- Hooker D* The development of reflexes in the mammalian fetus *Anat Rec* 1937 70 suppl 55
- Horsburgh D B and J P Heath* Atlas of cat anatomy Stanford California Stanford University Press 1950
- Hunt C C and A K McIntyre* Properties of cutaneous touch receptors in cat *J Physiol (Lond)* 1960 a 153 88—98
- An analysis of fibre diameter and receptor characteristics of myelinated cutaneous afferent fibres in cat *J Physiol (Lond)* 1960 b 153 99—112
- Hursh J B* Conduction velocity and diameter of nerve fibres *Am J physiol* 1939 a 127 131—139
- The properties of growing nerve fibres *Am J physiol* 1939 b 127 140—153
- Hyvarinen J* Analysis of spontaneous spike potential activity in developing rabbit diencephalon *Acta physiol scand* 1966 68 suppl 278
- Isigo A* New specific sensory structures in hairy skin *Acta Neuroveg* 1963 a 24 175—180
- An electrophysiological analysis of afferent fibres in primate skin *Acta Neuroveg* 1963 b 24 225—240
- Cutaneous receptors with a high sensitivity to mechanical displacement In Touch heat and pain Ciba foundation symposium 1965 Ed A V S de Reuck and J Knight 1966 237—256
- Job C* Über autogene Inhibition und Reflexumkehr bei spinalisierten und decerebrierten Katzen *Pflügers Arch ges Physiol* 256 406—418
- Kingsbury B F* The law of differential growth in its application to the nervous system *J Comp Neurol* 1932 56 431—464
- Klessens J J H M* Die Lage der Dermatome bei der Katze mittels des Strychnin Isolations Verfahrens bestimmt *Folia neuro biol Lpz* 1914 8 713—834
- Kugelberg E and A E Hagbarth* Spinal mechanism of the abdominal and erector spinae skin reflexes *Brain* 1958 81 290—304

- Kugelberg E K Eklund and L Grumby* An electromyographic study of the nociceptive reflexes of the lower limb Mechanism of the plantar responses Brain 1960 83 394—410
- Kuhn R A* Organization of tactile dermatomes in cat and monkey J Neurophysiol 1953 16 169—182
- Langworthy O R* The development of progression and posture in young opossums Am J physiol 1925 74 1—13
- Relation of onset of decerebrate rigidity to the time of myelination of tracts in the brain stem and spinal cord of young animals Contr Embryol Carnegie Inst Wash 1926 17 125—140
- A correlated study of the development of reflex activity in fetal and young kittens and the myelination of tracts in the nervous system Contr Embryol Carnegie Inst Wash 1929 20 127—171
- Lloyd D P C* Neuron patterns controlling transmission of ipsilateral hindlimb reflexes in cat J Neurophysiol 1943 6 293—315
- Spinal mechanisms involved in somatic activities In Handbook of Physiology — Neurophysiology II Ed J Field Baltimore Williams and Wilkins 1960 929—949
- Lundberg A* Alternative pathways from primary afferents to motoneurons In The effect of use and disuse on neuromuscular functions Proc of Liblice Symposium 1962 Ed E Gutmann and P Hnik Prague Pub House of Czech Acad of Sciences 1963 323—328
- Integration in the reflex pathway In Muscular afferents and motor control Nobel Symposium I 1965 Ed R Granit Stockholm 1966 275—305
- Malcolm J L* The development of reflex activity in the newborn kitten Abstr 19th Int Physiol Congr Montreal 1953 p 586
- The appearance of inhibition in the developing spinal cord of kittens In Biochemistry of the developing nervous system New York Academic Press 1955 104—109
- Maruhashi J K Miuguchi and I Tasaki* Action currents in single afferent nerve fibres elicited by stimulation of the skin in the toad and the cat J Physiol (Lond) 1952 117 129—151
- Megirian D* Bilateral facilitatory and inhibitory skin areas of spinal motoneurons of cat J Neurophysiol 1962 25 127—137
- Minkowski M* Sur les mouvements les reflexes et les reactions musculaires du foetus humain de 2 a 5 mois et leur relations avec le systeme nerveux foetal Rev neurol 1921 a 37 1105—1118 1235—1250
- Ueber Bewegungen und Reflexe des menschlichen Foetus während der ersten Hälfte seiner Entwicklung Schweiz Arch Neurol Psychiat 1921 b 8 148—151
- Zur Entwicklungsgeschichte Lokalisation und Klinik des Fusssohlenreflexes Schweiz Arch Neurol Psychiat 1923 13 475—514
- Mårtensson A* Proprioceptive impuls patterns during contraction of laryngeal muscles Acta physiol scand 1964 62 176—194
- Nomina Anatomica* Int Anat Nomenclature committee Excerpta Medica Foundation Amsterdam 1964

- Nyström B* and *S Skoglund* Calibre spectra of spinal nerves and roots in new born man. *Acta Morphol Neerland-Scand* 1965 6 115—127
- Pubols B H Jr* *W J Welker* and *J I Johnson Jr* Somatic sensory representation of forelimb in dorsal root fibres of racoon *coati mundi* and cat *J Neurophysiol* 1965 28 312—341
- Puletti F* Cutaneous tactile units in cat *Physiologist* 1959 2 96
- Ramón y Cajal S* Sur les fibres nerveuses de la couche granuleuse du cervelet et sur l'évolution des éléments cérébelleux *Int Mschr Anat Physiol* 1890 a 7 12—31
- Sur l'origine et les ramifications des fibres nerveuses de la moelle embryonnaire *Anat Anz* 1890 b 5 85—95
- Reighard J* and *H S Jennings* *Anatomy of the cat* New York Holt Rinehart and Winston 1961
- Rexed B* Contribution to the knowledge of the postnatal development of the peripheral nervous system in man *Acta psychiat et neurol* 1944 suppl 33
- Romero C* and *S Skoglund* Methodological studies of the technique in measuring nerve fibre diameters *Acta Morphol Neerland-Scand* 1965 6 107—114
- Scott P P* *A C da Silva* and *M A Lloyd Jacob* *The cat* In the UFAW handbook on the care and management of laboratory animals Ed A N Worden and W Lane Petter London UFAW 1957
- Sjöstrand F* The ultrastructure of the outer segments of rods and cones of the eye as revealed by electron microscope *J Cellular Comp Physiol* 1953 42 15—44
- Skoglund S* Anatomical and physiological studies of knee joint innervation in the cat *Acta physiol scand* 1956 36 suppl 124
- On the postnatal development of postural reflexes as revealed by electromyography and myography in decerebrate kittens *Acta physiol scand* 1960 a 49 299—317
- The spinal transmission of proprioceptive reflexes and the postnatal development of conduction velocity in different hindlimb nerves in the kitten *Acta physiol scand* 1960 b 49 318—329
- The activity of muscle receptors in the kitten *Acta physiol* 1960 c 50 203—221
- Central connections and functions of muscle nerves in the kitten *Acta physiol scand* 1960 d 50 222—237
- The reactions to tetanic stimulation of the two-neuron arc in the kitten *Acta physiol scand* 1960 e 50 238—253
- Unpublished observations 1963
- Muscle afferents and motor control in the kitten In *Muscular afferents and motor control* Nobel symposium I 1965 Ed R Granit Stockholm 1966 245—259
- Unpublished observations 1967
- Skoglund S* and *C Romero* Postnatal growth of spinal nerves and roots A morphological study in the cat with physiological correlations *Acta physiol scand* 1965 66 suppl 260
- Stowell T B* The vagus nerve in the domestic Cat *Proc Am Philos Soc* 1882 20 123—138

- Stowell T B* The trigeminal nerve in the domestic cat *Proc Am Philos Soc* 1886 23 459—478
- The glossopharyngeal accessory and hypoglossal nerves in the domestic cat *Proc Am Philos Soc* 1887 24 89—104
- The lumbar the sacral and the coccygeal nerves in the domestic cat *J Comp Neurol* 1891 1 287—358
- Szabó G* The regional frequency and distribution of hair follicles in human skin In *The biology of hair growth* Ed W Montagna and R A Ellis New York Academic Press 1958 33—38
- Tapper D N* Cutaneous slowly adapting mechanoreceptors in the cat *Science* 1964 143 53—54
- Thomas C E and C M Combs* Spinal cord segments A Gross structure in the adult cat *Am J Anat* 1962 110 37—47
- Wall P D* Cord cells responding to touch damage and temperature of skin *J Neurophysiol* 1960 23 197—210
- Weed L H* The reactions of kittens after decerebration *Am J physiol* 1917 43 131—157
- Werner G and V B Mountcastle* Neural activity in mechanoreceptive cutaneous afferents stimulus — response relations, Weber functions and information transmission *J Neurophysiol* 1965 28 359—397
- Wilson V J* Reflex transmission in the kitten *J Neurophysiol* 1962 25 263—276
- Windle W F* The relation of the level of transection of the brain stem to the occurrence of decerebrate rigidity in young animals *J Comp Neurol* 1929 48 227—245
- Normal behavioral reactions of kittens correlated with the postnatal development of nerve fibre density in the spinal gray matter *J Comp Neurol* 1930 50 479—504
- Correlation between the development of local reflexes and reflex arcs in the spinal cord of cat embryos *J Comp Neurol* 1934 59 487—505
- Physiology of the fetus Origin and extent of function of prenatal life Philadelphia Saunders 1940
- Windle W E and A M Griffin* Observations on embryonic and fetal movements of the cat *J Comp Neurol* 1931 52 149—188
- Witt I and H Hensel* Afferente Impulse aus der Extremitätenhaut der Katze bei thermischer und mechanischer Reizung *Pflug Arch* 1959 268 582—596
- Yamamoto S S Sugihara and M Kuru* Microelectrode studies on sensory afferents in the posterior funiculus of cat *Jap J Physiol* 1956 6 68—85
- Zotterman Y* Touch pain and tickling an electrophysiological investigation on cutaneous sensory nerves *J Physiol (Lond)* 1939 95 1—28





ACTA PHYSIOLOGICA SCANDINAVICA

**STUDIES ON THE METABOLISM OF  
1,4-<sup>14</sup>C-SPERMIDINE AND 1,4-<sup>14</sup>C-  
SPERMINE IN THE RAT**

BY

MARTTI SIIMES

HELSINKI 1967





ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 298

FROM THE DEPARTMENT OF MEDICAL CHEMISTRY  
UNIVERSITY OF HELSINKI FINLAND

**STUDIES ON THE METABOLISM OF  
1,4-<sup>14</sup>C-SPERMIDINE AND 1,4-<sup>14</sup>C-  
SPERMINE IN THE RAT**

BY

MARTTI SIIMES

HELSINKI 1967



## PREFACE

The present work was carried out in the Department of Medical Chemistry University of Helsinki. I am very much indebted to my honoured chief Professor JOHAN JÄRNEFELT, MD for his unfailing interest and advice during the work and for his kindness in placing all the facilities of his department at my disposal.

My heartfelt gratitude is due to Dr AARNE RAINA MD for his unfailing teaching tuition which led me to adopt the subject of this investigation for his beneficial advice and discussions which made my work possible and for his interest and most truebred criticism and guidance during the work.

I am also greatly indebted to Assistant Professor MARTTI KOIVUSALO MD and Docent NIELS RÄIHÄ MD for their encouraging criticism of the manuscript.

I further wish to thank my colleagues in the department especially my great friend Dr JUHANI JÄNNE for his valuable help and Dr JAAKKO PISPA MD for his advice during the work.

The English text of my manuscript has been revised by Miss ANN MATHESON MA whom I should like to thank. Further I wish to thank Mrs SIRKKA KANERVA for her skilful laboratory assistance and Miss MARIA SAARILUOMA for carrying out the secretarial work.

And aboveall I wish to thank my wife ANJA SIIMES BM for her constant understanding and support during this work.

Helsinki April 1967

MARTTI SIIMES



# CONTENTS

INTRODUCTION	7
MATERIALS	13
METHODS	13
EXPERIMENTAL PART	21
Fate of 14 <sup>14</sup> C polyamines in tissues	21
Distribution of 14 <sup>14</sup> C polyamines in tissues	21
Disappearance of 14 <sup>14</sup> C polyamines in tissues	25
Excretion of radioactivity derived from 14 <sup>14</sup> C polyamines	28
Excretion into urine	28
Excretion into intestine	29
Degradation into carbon dioxide	31
Recovery of injected radioactivity	32
Interconversion of 14 <sup>14</sup> C polyamines in tissues	32
Incorporation of 14 <sup>14</sup> C spermidine into spermine in liver	32
Incorporation of 14 <sup>14</sup> C spermine into spermidine in liver	35
Interconversion of 14 <sup>14</sup> C polyamines in different tissues in relation to age	37
Incorporation studies with 14 <sup>14</sup> C polyamines during liver regeneration	41
Interconversion of 14 <sup>14</sup> C polyamines during ethionine treatment	45
Changes in liver polyamines during starvation	47
DISCUSSION	50
SUMMARY	59
REFERENCES	61



## INTRODUCTION

The polyamines spermidine  $\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$  and spermine  $(\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2)$  are aliphatic nitrogenous bases widely distributed in various organisms. The existence of polyamines has been known for a long time probably since the 17th century (LEWENHOECK 1678). Only during the last decade has an increasing amount of knowledge been accumulated about these compounds. In this context some light will be thrown on the occurrence, possible functions, biosynthesis and degradation of polyamines.

**Occurrence of polyamines** Even as early as 1925 DUDLEY and ROSENHEIM determined the spermine of several animal tissues. Human semen, ox liver, kidneys and spleen were reported to contain relatively large amounts of spermine. ROSENTHAL and TABOR (1956) analysed both the spermidine and spermine contents of various organs in a number of laboratory animals. A detailed study of the concentrations of polyamines in different rat tissues was reported by JÄNNE, RAINA and SIIMES (1964). In three month old rat the concentration of spermidine per gram of tissue wet weight varied in different tissues from 100 to 1 600  $\mu\text{moles}$  and that of spermine from 160 to 900  $\mu\text{moles}$ . The authors also observed in every studied tissue a decrease in the spermidine concentration in relation to age, e.g. from 2 000 to 600  $\mu\text{moles}$  per gram of liver from birth to nine months. The fall was most marked during the first month of life. In some tissues there was a rise in the spermine concentration during the first month but in general the changes in this amine with age were small. Recently the brain polyamine contents of various mammals have also been analysed (JÄNNE, RAINA and SIIMES 1964; SHIMIZU, KAKIMOTO and SANO 1964; PERRY, HANSEN, FOULKS and LING 1965; MICHAELSON 1966). A decrease in the spermidine concentration of mouse brain during the perinatal period was established by SHIMIZU, KAKIMOTO and SANO (1965a). Only small amounts of polyamines, about five  $\mu\text{moles}$  per millilitre, have been observed in the blood cells, as pointed out by RAINA (1962a) and SHIMIZU, KAKIMOTO



and SANO (1965b) and none in human blood plasma (ROSENTHAL and TABOR 1956 RAINA 1962a BACHRACH and ROBINSON 1965)

Relatively high concentrations of polyamines and especially putrescine a spermidine precursor have been observed in various microorganisms *E coli* has been reported to contain 15  $\mu$ moles of putrescine and 15  $\mu$ moles of spermidine per gram (TABOR ROSENTHAL and TABOR 1958) However the polyamine concentrations in different bacteria vary with the pH and other cultural conditions (TABOR ROSENTHAL and TABOR 1958, DUBIN and ROSENTHAL 1960a RAINA and COHEN 1966) A great variety of polyamine is found in different strains Part of the putrescine and spermidine are in the acetylated forms (DUBIN and ROSENTHAL 1960a, RAINA and COHEN 1966 COHEN and RAINA 1967) It is noteworthy that the T even type bacteriophages of *E coli* contain very high concentrations of putrescine and spermidine per gram (AMES and DUBIN 1960) In addition various plants are known to contain polyamines as discussed by TABOR and TABOR (1964b)

**Possible functions of polyamines** Several authors have made efforts to find some function for polyamines in cells In 1949 HERBST and SNELL were the first to find that spermine is a growth factor for *Hemophilus parainfluenzae* Even now the mechanism by which spermine influences growth is not definitely established On the other hand spermine is also known to inhibit both the growth (RAZIN and ROZANSKY 1959) and the protein synthesis of *Staphylococcus aureus* (FRIEDMAN and BACHRACH 1966) and of Walker carcinosarcoma cells (GOLDSTEIN 1965) The studies *in vitro* have shown that spermine spermidine and putrescine stimulate the incorporation of amino acid into protein (HERSHKO AMOZ and MAGER 1961 MARTIN and AMES 1962) possibly as a result of the stabilization of 70S ribosomes by polyamines (ZILLIG KRONE and ALBERS 1959 COHEN and LICHTENSTEIN 1960 COLBOURN WITHERSPOON and HERBST 1961 MARTIN and AMES 1962) in the presence of suboptimal magnesium ion concentration (HERSHKO AMOZ and MAGER 1961) It has also been demonstrated that at low concentrations in the medium spermine stimulates and at high concentration inhibits polypeptide synthesis by a subcellular system derived from L 1210 mouse ascites leukemia (OCHOA and WEINSTEIN 1964) The inhibitory effect of spermine is dependent upon the concentrations of both rRNA and mRNA but not of microsomes (OCHOA and WEINSTEIN 1965)

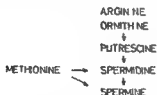
Many close associations have been reported between polyamines and nucleic acids. Polyamines precipitate nucleic acids *in vitro* (TABOR 1962). They have the ability of protecting helical DNA against thermal denaturation (MANDEL 1962). In the presence of polyamines an increased resistance to thermal denaturation of RNA has also been noticed (GOLDSTEIN 1966). The polyamines have a strong affinity with the cellular constituents of acidic groups. A number of reports indicate that polyamines can stabilize and protect ribosomes, mitochondria, cell membranes, bacteriophages and nucleic acids themselves. The association between polyamines and cellular structures and nucleic acids has been discussed in detail in a review by TABOR and TABOR (1964b) and very recently by COHEN and RAINA (1967). The latest studies cited below have thrown considerable light on the role of polyamines in relation to nucleic acids. RAINA and COHEN (1966) observed that the ratio of total polyamine nitrogen to RNA phosphate varied within fairly narrow limits (0.21–0.23) in growing cells of *E. coli*. Some studies have shown that spermine, spermidine and putrescine *in vitro* somewhat stimulate the initial rate of RNA synthesis and allow also the action of RNA polymerase to continue (KRACKOW 1963, FOX, ROBINSON, HASELKORN and WEISS 1964, FOX and WEISS 1964). The DNA primed RNA polymerase reaction was progressively stimulated by graded concentrations of spermine to  $10^{-4}$ M (O'BRIEN, OLENICK and HAHN 1966). The effect *in vivo* might be similar. In fact this kind of effect has been demonstrated by RAINA and COHEN (1966). The exogenous putrescine 150 mM caused a slight increase and the exogenous spermidine 20 mM or more a marked increase in the uracil incorporation into RNA. These direct observations on the effects of polyamines were made in the polyauxotrophic strain of *E. coli* during an amino acid starvation. A rise in the RNA synthesis has also been obtained by spermine in *E. coli* (MILLS and DUBIN 1966) and in a tissue culture (GOLDSTEIN 1965). Recently SIMON, COHEN and RAINA (1966) studied the inhibition of RNA synthesis in *E. coli* and they noticed that spermidine reversed the inhibition of RNA synthesis caused by levorphanol.

Spermine ( $10^{-4}$ M) has been demonstrated as an inhibitor of the DNA polymerase activity *in vitro* (O'BRIEN, OLENICK and HAHN 1966). The latest observations by BREWER and RUSCH (1966) indicate the stimulatory effect of spermine when administered together with DNA on the DNA polymerase reaction in the isolated nuclei of a plasmodia. This effect was most noticeable in the nuclei isolated 1.5 hours after mitoses.

Only a slight stimulation was obtained with spermine and DNA used separately under the same experimental conditions

**Biosynthesis of polyamines** The first experiments concerning the biosynthetic pathways of polyamines in microorganism were reported in 1956–1958. Labelled putrescine and ornithine were shown to serve as precursors of the 4 carbon chain (TABOR ROSENTHAL and TABOR 1956 1958) and methionine of the 3 carbon moieties in spermidine and spermine (GREENE 1957 TABOR ROSENTHAL and TABOR 1957 1958). In this reaction methionine is used in the form of S-adenosyl methionine (CANTONI and DURELI 1957) which is decarboxylated for the spermidine synthesis (TABOR ROSENTHAL and TABOR 1956 1957 TABOR and TABOR 1960 TABOR 1962a b) MORRIS and PARDEE (1965 1966) have shown that biosynthesis of putrescine occurs directly through the decarboxylation of ornithine or through the formation of agmatine from arginine in *E. coli*. Purification of the enzymes catalysing the steps in the spermidine biosynthesis from putrescine and methionine has also been reported by TABOR and TABOR (1960) and TABOR (1962 a b). On the other hand spermidine could not substitute for putrescine as an acceptor of propylamine moiety (TABOR 1962b). Accordingly nothing is known about the enzymatic mechanisms in the biosynthesis of spermine from spermidine.

Polyamine biosynthesis has also been demonstrated by  $^{14}\text{C}$  labelled precursors in chick embryo by RAINA (1962b 1963) and recently in rat tissues by RAINA (1964) JÄNNE and RAINA (1966) and RÄIHÄ JÄNNE and SUIHKONEN (1967). The same precursors were described in rat tissues as in microorganisms illustrated as follows



The synthesis of polyamines has also been studied in rat liver regenerating after partial hepatectomy. The incorporation of radioactivity from labelled arginine (JÄNNE and RAINA 1966) and especially from methionine (RAINA JÄNNE and SIIMES 1966) into spermidine has been shown to increase greatly in the liver after partial hepatectomy. DYKSTRA and HERBST (1965) reported that the conversion of  $^3\text{H}$  putrescine into sper

spermidine in the regenerating rat liver was almost double as early as two hours after operation. However JÄNNE and RAINA (1966) pointed out that there was a fall in the specific activity of spermidine after administration of labelled putrescine. RAINA, JÄNNE and SIIMES (1965, 1966) and DYKSTRA and HERBST (1965) observed an increase in the spermidine concentration of the liver from most noticeable 64 to 72 hours after partial hepatectomy. A decrease in the spermidine concentration per gram of liver at 32 hours was also demonstrated. The ratio of total spermidine-nitrogen to ribonucleic acid phosphate appeared to be quite constant during liver regeneration. There was also a correlation between the concentrations of deoxyribonucleic acid and spermidine throughout the period of regeneration (RAINA, JÄNNE and SIIMES 1965, 1966). The miscellaneous changes in the metabolism of regenerating liver have been reviewed by BUCHER (1963).

Ethionine is a toxic methionine analogue which changes the metabolism of tissues in many respects. It has been reported to inhibit the synthesis of RNA (VILLA TREVINO, SHULL and FARBER 1963b; FARBER, SHULL, VILLA TREVINO, LOMBARDI and THOMAS 1964) and in addition stimulation effects have also been shown (TURNER and REID 1964). In its role ethionine is known to inhibit protein synthesis, lower the activities of several enzyme systems and cause hepatomas etc. The interesting material concerning the effects of ethionine on tissues has been reviewed by STEKOL (1963).

Ethionine being a methionine antagonist has been used to obtain information on the metabolism of polyamines. RAINA (1963) reported that ethionine treatment reduced both the spermidine concentration and the incorporation of labelled methionine into this polyamine in the developing chick embryo. During daily ethionine administrations the concentration of spermidine rises in rat liver (RAINA, JÄNNE and SIIMES 1964; KREZDNER and STARR 1966) and that of spermidine decreases (RAINA, JÄNNE and SIIMES 1964). These effects have been attributed to the formation of S-adenosylethionine (MODY, BULBA, HOLOWECKY and STEKOL 1963; RAINA, JÄNNE and SIIMES 1964; SHULL, McCONOMY, VOGT, CASTILLO and FARBER 1966) with a fall in the adenosyl nucleotides (STEKOL, MODY, BEDRAK, KELLER and PERRY 1960; CALDARA, BUDINI, BARBIROLI and RABBI 1962; SHULL 1962; VILLA TREVINO, SHULL and FARBER 1963a; BARTELS and HOHORST 1963; MODY, BULBA, HOLOWECKY and STEKOL 1963; RAINA, JÄNNE and SIIMES 1964). The effect of ethionine on the concentrations of polyamines

is dependent upon the daily dosage the cessation of treatment and the tissue which has been analysed (SIIMES RAINA and JÄNNE und r preparation)

**Degradation of polyamines** HIRSCH (1953a b) was the first to discover in the blood plasma of the sheep and the ox an enzyme which caused rapid oxidative deamination of spermine and which also acted on spermidine. He called this enzyme spermine oxidase. Using enzyme preparations it was reported that spermine is first converted into spermidine which is then further oxidized (TABOR and ROSENTHAL 1956b BACHRACH and BAR OR 1960 YAMADA and YASUNOBY 1962 UNEMOTO 1963). The formation of ammonia hydrogen peroxide and aldehydes during oxidation was also demonstrated (ROULET and ZELLER 1945 HIRSCH 1953a b TABOR and ROSENTHAL 1954). Recently TABOR TABOR and BACHRACH (1964) have shown using purified enzyme preparations that polyamine aldehydes are the only products of this oxidation. This discovery does not agree however with those mentioned above i.e. that free spermidine is formed from spermine by plasma oxidase.

As early as 1944 SILVERMAN and EVANS noticed that polyamines were rapidly oxidized by *Pseudomonas aeruginosa*. Some reports show that various bacteria are able to degrade spermine to spermidine (RAZIN BACHRACH and GERY 1958 RAZIN GERY and BACHRACH 1959 BACHRACH PERSKY and RAZIN 1960). A recent report indicates that spermidine is further degraded to putrescine and 3 aminopropionaldehyde in *Pseudomonas aeruginosa* (PAD MAHABHAN and KIM 1965). BACHRACH (1962 a b) purified an enzyme from *Serratia marcescens* which oxidized spermidine to 1,3 diaminopropane and  $\Delta^1$  pyrroline. This enzyme did not act on spermine and it was reported to be different from the plasma oxidase.

The nonoxidative metabolism of polyamines in bacteria has also been studied. Acetylation of polyamines has been pointed out by DUBIN and ROSENTHAL (1960) ROSENTHAL and DUBIN (1962) and RAINA and COHEN (1966). The products are in *E. coli* monoacetylputrescine two isomeric forms of monoacetylspermidine derived from spermidine and mono- and diacetylspermine from spermine (DUBIN and ROSENTHAL 1960). The glutathione conjugates of the polyamines have also been reported (DUBIN 1959 DUBIN and ROSENTHAL 1960 b TABOR TABOR and de MEIS 1966).

Little information is available on the degradation of polyamines in

animal tissues. The only evidence for the degradation of spermine to spermidine in animals has been observed by ROSENTHAL and TABOR (1956) when they studied the distribution and excretion of polyamines. After the administration of nonlabelled spermine to rats, mice and rabbits 4 to 8 per cent of the dose was excreted in the urine as spermidine during the first day. When spermidine was injected, no spermine appeared in the urine. The concentration of spermidine was also found to increase slightly in a few tissues after administration of spermine.

More detailed knowledge of the metabolic role of polyamines and their metabolism has been reviewed by TABOR, TABOR and ROSENTHAL (1961), BLASCHKO (1962), RAINA (1963), TABOR and TABOR (1964), BEER, ROSIEK and SABLINSKY (1966) and COHEN and RAINA (1967).

The purpose of the present investigation was to study the metabolic fate of exogenously administered spermidine and spermine in rat tissues by  $1,4\text{-}^{14}\text{C}$  labelled polyamines. The study of polyamine interconversion in rat tissues *in vivo* formed the main part of this work. Evidence on the transformation of polyamines has been reported in bacteria, but in animal tissues attention has not been paid to this section of polyamine metabolism.

The polyamine concentrations in the rat tissues are dependent upon the tissue and the age of the rat. Relatively high spermidine concentrations have been noticed in every young rat tissue studied. The concentration of spermidine also varies considerably in different tissues obtained from rats of the same age. For instance, the concentration of spermidine and the molar ratio spermidine/spermine is fairly low in the kidneys and unusually high in the pancreas. This data prompted a study of polyamine interconversion in relation to age in the pancreas and kidneys as well as in the liver and intestine.

An earlier study of the biosynthesis of polyamines has also been made in regenerating rat liver after partial hepatectomy. During the development of regeneration, the biosynthesis and the concentration of spermidine is known to be increased. In addition, the treatment with ethionine causes characteristic changes in the polyamine concentrations. These variables also formed a basis for the study of the interconversion of administered polyamines.

---

\*) In literature not only spermidine and spermine are regarded as polyamines but often also diamines such as putrescine and cadaverine. However, in this text only spermidine and spermine will be referred as polyamines.

In this study the radioactive amines were given exogenously to the rats. To obtain a picture which would be as close as possible to the metabolism of the endogenous polyamines, only trace amounts of these compounds with high specific activity were used. This handling did not essentially change the pool of endogenous polyamines. On the other hand, there is only poor evidence on whether the metabolism of exogenous polyamines, besides those of synthesized forms, is an essential element in the total metabolism of polyamines, e.g. by absorbing from the intestine.

Knowledge on the turnover of exogenously administered polyamines is also lacking. Accordingly, it was considered necessary to study also the distribution and elimination of exogenously injected polyamines in the tissues and their possible routes of elimination in rat organisms, as for example, through the lungs into the urine and into the intestine. It was hoped that a better understanding of the fate of injected  $^{14}\text{C}$  labelled polyamines would afford some more information on the metabolism and the metabolic role of these amines.

## MATERIALS

**Animals** The rat was chosen as the experimental object for the present study. The biosynthetic pathways of polyamines in rat tissues have recently been studied in this laboratory. This animal was thus considered to be suitable for use in the study of the fate of these amines. The experiments were performed *in vivo* with female Wistar strain rats. The age of the animals varied from the newborn to 30 weeks. The newborn rats were less than 6 hours old at the commencement of the experiment. The animals were fed unless otherwise indicated *ad libitum* with a standard diet manufactured by Hankkija Oy, Helsinki, Finland. Variations in the weight of rats in different groups was kept small and it never exceeded  $\pm 10$  per cent of the mean value. Each group comprised 2 to 8 rats and normally 4 in the isotope experiments.

**Reference standards** Spermidine phosphate, spermine tetrahydrochloride and putrescine dihydrochloride were commercial analytical grade preparations from Mann Research Laboratories Inc., New York, U.S.A.

**Reagents** The reagents and solvents used in these experiments were of analytical grade. They were mainly manufactured by E. Merck, Darmstadt, Germany.

**Isotopes** Two  $^{14}\text{C}$ -labelled compounds now commercially available were used: Spermidine 1,4- $^{14}\text{C}$  tetrahydrochloride [(aminopropyl) tetramethylene-1,4- $^{14}\text{C}$  diamine]  $\cdot 4\text{HCl}$  and spermine 1,4- $^{14}\text{C}$  tetrahydrochloride [(bis (aminopropyl) tetramethylene 1,4- $^{14}\text{C}$ -diamine]  $\cdot 4\text{HCl}$  were both purchased from the New England Nuclear Corporation, Boston, Massachusetts, U.S.A. The specific activity of spermidine was 66 mCi/mmole and that of spermine 4.2 mCi/mmole. The stock isotope was stored at a temperature of  $-18^\circ\text{C}$ .

## METHODS

### Handling of the animals

Labelled polyamines were usually injected intraperitoneally to the rats. Subcutaneous and oral administration were also used in a few experiments. The injections were made in a volume of 0.2 to 1.0 ml in 0.9 per cent sodium



chloride The rats were killed by decapitation The tissues were weighed in cool and homogenized in 0.1 N hydrochloric acid The details of the method of preparation and handling of the tissues have been described previously by RAINA (1963)

#### Collection of the urine

The urine was collected from the rats fed *ad libitum* on a porcelain plate under the cage Before collection about 10 ml of 0.1 N hydrochloric acid was placed on the plate which was covered by a metal web to prevent a contamination by feces The urine which has been diluted with hydrochloric acid was filtered and evaporated to a volume of 8 ml at room temperature These samples were treated in a similar way as the tissue homogenates (RAINA 1963)

#### Collection of carbon dioxide

Respiratory  $\text{CO}_2$  was collected and its radioactivity measured after the injection of labelled polyamines to rats A rat fed *ad libitum* was placed immediately after injection in a glass jar into which incoming air came through four washing tubes each containing 50 ml of 1 N sodium hydroxide The exhaled  $\text{CO}_2$  was collected in five similar tubes by water suction After collection which lasted for 1 to 3 hours the sodium bicarbonate in these five tubes was combined and mixed An aliquot of 5 ml was placed in an Erlenmeyer flask with a centre well In addition 1 ml of hyamine (Packard Instrument Company Inc Illinois USA) was placed in the centre wells The flasks were tightly closed with rubber stoppers 2 ml of 4 N hydrochloric acid was injected through the stoppers into the sodium bicarbonate solution to liberate  $\text{CO}_2$  Twelve flasks at a time were slowly shaken in a mechanical shaker for one hour at room temperature The hyamine was transferred into 9 ml of toluene scintillation liquid with which the centre wells were carefully rinsed several times The controls without radioactivity were treated in a similar way Freshly prepared sodium hydroxide and hyamine were used

#### Partial hepatectomy

Partial hepatectomy of the rats was performed according to HIGGINS and ANDERSON (1931) The rats were anaesthetized with aether A single thread ligature was tied few millimetres distally from the pedicles and the lobes removed These three liver lobes together amounted to about two-thirds of the liver weight The operation appeared to have no fatal results if properly performed The abdominal cavity was closed tightly in two layers After decapitation when the residual livers were analysed only the two corresponding lobes were analysed from both the operated and control rats

## Quantitative determination of polyamines and putrescine

Tissue polyamines were separated by paper electrophoresis of an alkaline butanol extract from the deproteinized sample. Amido black was used as the colour reagent. This sensitive method has been described by RAINA (1963). Putrescine was analysed quantitatively from the same tissue samples according to RAINA and COHEN (1960) using cadmium ninhydrin as the colour reagent.

### Radioactivity measurements

The radioactivity in putrescine, spermidine and spermine can be separated by electrophoresis on paper (RAINA 1963). The separation of polyamines for radioactivity measurements was obtained with 0.1 M citric acid buffer pH 3.6 in 3 hours and that of putrescine at pH 4.3 in 2 hours. The fractions were visualized by spraying with 0.1 per cent ninhydrin reagent. From the paper strips the polyamine fractions and 2 blank fractions were cut out, one blank far from the cathode side of the paper and one between the two polyamine fractions. The pieces of papers containing the radioactivities of the polyamines and blank fractions were placed directly in the scintillation flasks. A day after the addition of 2 ml of scintillation liquid (3 g 2,5-diphenyloxazole and 0.1 g 1,4-bis-(4-methyl-phenyloxazolyl) benzene/1000 ml toluene) obtained from Packard Instrument Company Inc. Illinois, USA, the radioactivity was measured in a Packard Tri Carb Liquid Scintillation Spectrometer Model III.

In this study 10  $\mu$ C of 1,4- $^{14}$ C spermidine standard on paper gave around 1,040,000 counts per min and 1,4- $^{14}$ C spermine about 1,080,000 counts per min. These standards were measured in a similar way and counted during every series of experiments.

All radioactivity measurements in this work were performed on the paper by the above mentioned technique except for that of carbon dioxide. The hyamine bound  $^{14}$ C  $\text{CO}_2$  was diluted with toluene scintillation liquid too but the counting efficiency was about 15 per cent higher in these measurements than in the measurements directly from the paper.

### Control of the radioactivity measurements

The paper electrophoretic technique produced good separation between spermidine and spermine radioactivities. During the optimal conditions of the routine method 1,4- $^{14}$ C spermidine diluted in 20  $\mu$ moles of spermidine and spermine produced a contamination of about 0.5 per cent in the fraction between polyamines and about 0.2 per cent in the spermine fraction relative to the radioactivity on spermidine. The contamination from the labelled spermine in the spermidine fraction was only 0.05 to 0.01 per cent of the radioactivity in the spermine fraction. These contaminations were considered to be without practical significance in the present work.

The Rf values of  $14^{14}\text{C}$  spermine and spermidine which were formed in the liver with some solvent systems as given in Table I were equal to those of the endogenous polyamine fractions

The only radioactive compounds were spermidine and spermine which were found in the liver samples one day after an intraperitoneal injection of a  $14^{14}\text{C}$  polyamine demonstrated as follows. According to the routine method described by RAINA (1963) the trichloroacetic acid supernatant of the tissue homogenate was extracted with aether 3 times. The aether extracts were combined, evaporated at room temperature and diluted to a small volume which was placed totally on the paper, dried and measured in toluene scintillation solution. Only trace of radioactivity was found in this sample. The trichloroacetic acid bound protein was diluted in sodium hydroxide and its radioactivity was measured in Bray's solution (BRAY 1960). Here again only small amounts of radioactivity were detected. As the next step, butanol extraction of the samples was performed from both alkaline and acid solution. The recovery of the radioactivity after the alkaline butanol extraction was essentially the same as reported by RAINA (1963). Only around 5 per cent of the radioactivity was recovered from the acid butanol. Alkaline butanol soluble radioactivity was found in the electrophoretic fractions of polyamines: spermidine and spermine.

The radiopurity of the spermidine and spermine fractions was also tested from the liver samples 2 days after injection of labelled spermidine or spermine as follows. The polyamines were separated by a preparative ascending paper chromatography with the solvent VII (see Table I). On the paper, narrow strips were stained with 0.1 per cent ninhydrin reagent using a micropipette. Following these small guide fractions, the unstained spermidine and spermine fractions were separately cut off and eluted. After alkaline butanol extraction (RAINA 1963), the samples were subjected to paper electrophoresis. After electrophoretic separation, the specific activities of spermine and spermidine were essentially the same as after the first separation by chromatography.

The radioactive spermidine derived from  $14^{14}\text{C}$  spermine and isolated by preparative paper electrophoresis as described above was also diluted with nonlabelled spermidine tetrahydrochloride. The preparation was recrystallized in ethanol/water (JACKSON and ROSENTHAL 1960). The precipitate was collected by centrifugation, washed several times with 99.9 per cent ethanol and dried in vacuum. The specific activity of spermidine was essentially the same at the beginning of the experiment as after the crystallization.

Further experiments were carried out to eliminate the possible occurrence of polyamine derivatives on the electrophoretic fractions of spermidine and spermine. RAINA (personal communication) has observed that acetyl derivatives of polyamines and putrescine, which are normal constituents in *E. coli*, run in the same area as polyamines themselves in the paper electrophoresis during the routine procedure. The radioactive polyamine fractions were separately hydrolysed in strong acid. The samples were kept in 6 N hydrochloric acid in sealed tubes at a temperature of 100 and 120°C for 12 hours. The specific activities of labelled polyamines, which were found in the liver, were essentially the same after the hydrolysis. Aldehyde derivatives of the polyamines were excluded

by treating the liver homogenate 1 and 2 days after and the blood 70 min after the intraperitoneal injections of  $14\text{C}$  polyamines with sodium borohydride reagent overnight. The details of this procedure have been described by TABOR TABOR and BACHRACH (1964). It is likely according to above mentioned

*Table 1 Chromatography of liver polyamines and putrescine. Rf values were determined by staining the chromatograms with ninhydrin or amido black (RAINA 1963) reagents. The samples were first run with paper electrophoresis or solvent VI in a thin layer. The amine fractions were then eluted and rerun with other solvents.*

System	Rf values		
	Spermone	Spermidine	Putrescine
Sol I <sup>1)</sup>	0.30 <sup>2)</sup>	0.60 <sup>2)</sup>	1.00 <sup>2)</sup>
Sol II <sup>3)</sup>	0.02	0.07	
Sol III <sup>3)</sup>	0.25	0.38	
Sol IV <sup>3)</sup>	0.18	0.25	0.34
Sol V <sup>3)</sup>	0.24	0.34	0.44
Sol VI <sup>4)</sup>	0.38	0.46	

Sol I n butanol glacial acetic acid water 50:25:25 (FINK CLINE and FINK 1963)

Sol II n butanol methylethylketone formic acid water 40:30:15:15 (FINK CLINE and FINK 1963)

Sol III iso propanol water conc. hydrochloric acid 65:18:4:16.6 (FINK CLINE and FINK 1963)

Sol IV n butanol acetic acid pyridine water 40:10:10:20 (DUBIN and ROSENTHAL 1960)

Sol V n propanol conc. hydrochloric acid water 30:10:10 (DUBIN and ROSENTHAL 1960)

Sol VI ethylene glycol monomethyl ether propionic acid water - 70:25:15 (RAINA 1963)

Sol VII <sup>3)</sup> sol VI in saturated sodium chloride (RAINA 1963)

<sup>1)</sup> Descending paper chromatography Whatman No 1 paper Running time 48 hours

<sup>2)</sup> Correlated with Rf values of putrescine which was run about a distance of 30 cm

<sup>3)</sup> Ascending paper chromatography with paper of Whatman No 1 Run a distance of 18 to 20 cm

<sup>4)</sup> Thin layer chromatography Kieselgel G 70 g - water 100 ml The gel was 0.5 mm thick Run a distance of 15 cm

evidences that there are no acetyl or aldehyde derivatives with the label in liver after injection of labelled polyamines

During the early regeneration of the liver the incorporation of  $14^{14}\text{C}$  spermidine into putrescine was stimulated. A few studies were carried out on the identification of the radioactivity of the electrophoretic fraction of putrescine. The used  $14^{14}\text{C}$  spermidine solution in 20  $\mu\text{moles}$  of putrescine spermidine and spermine about  $10^6$  cpm per electrophoretic paper strip gave practically no contamination on the putrescine fraction. In the normal liver 3 hours after an injection of  $14^{14}\text{C}$  spermidine ( $5 \times 10^5$  cpm) the radioactivity on the putrescine fraction barely exceeded the background level. Six hours after partial hepatectomy when the similar injection had been given 3 hours earlier the counts on putrescine were about 4 fold the background. From the pooled sample derived from the partially hepatectomized livers after injection of labelled spermidine the putrescine fraction was separated from the polyamines by a preparative chromatography with solvent VII see Table I. The  $R_f$  values of the putrescine fraction are given with 3 solvent systems of different chromatographies in Table I. No loss of counts compared to the primary paper electrophoretic fraction was found in the putrescine of the chromatographies. The sodium borohydride treatment (TABOR, TABOR and BACHRACH 1964) did not show any effect on the counts in the putrescine fraction either.

*Statistical procedures* In general the presented values are arithmetic means. In some instances also the range of values in a group is given. The detailed statistical analyses were not considered essential in this study because the variation in the values inside a group was small enough to show the differences found. Usually the deviation in the separate values was less than  $\pm 10$  per cent of the mean. In some experiments demonstrating the results of ethionine treatment carbon dioxide collection radioactivities in the blood and absorption from the intestinal tract somewhat larger deviations were noticed. They were mostly less than  $\pm 20$  per cent of the mean value of a group.

## EXPERIMENTAL PART

### FATE OF $14^{14}\text{C}$ -POLYAMINES IN TISSUES

#### DISTRIBUTION OF $14^{14}\text{C}$ -POLYAMINES IN TISSUES

Some aspects of the metabolism of polyamines in the organism may be conveniently studied by following the fate of the exogenously administered labelled polyamines. First the total distribution of the injected polyamines was studied in some tissues. Comparisons were also made using different routes of administration.

The distribution of  $14^{14}\text{C}$ -spermidine in certain tissues after intraperitoneal injection is presented in Table II.  $1.0 \mu\text{C}$   $14^{14}\text{C}$ -labelled spermidine (about  $10^6$  cpm) was injected into one and two month old rats and they were decapitated 12 hours after injection. Table II indicates that a great part 71.6 per cent of the injected radioactivity was still unchanged as  $^{14}\text{C}$  spermidine in the whole animal 12 hours after an

*Table II Distribution of injected  $1.4^{14}\text{C}$  spermidine in tissues. Spermidine radioactivities per organ and a percentage of the injected dose have been determined 12 hours after an injection. The dose was  $1.0 \mu\text{C}$  (about  $10^6$  cpm) per rat. The age of the rats was one month. Means and ranges of 4 animals in a group are presented.*

Tissue	Distribution of $1.4^{14}\text{C}$ spermidine	
	cpm per organ	per cent of dose
Liver	207 000 (182 000 — 231 000)	20.0
Kidneys	18 600 ( 16 500 — 20 000)	1.8
Intestine	286 000 (234 000 — 305 000)	27.6
Rest of tissues	230 000 (198 000 — 242 000)	22.2
Total	741 000	71.6

injection. Nearly one half of the given activity was recovered merely from the liver and intestine although the total weight of these two tissues represented about 15 per cent of the rat weight. 18 per cent was found in the kidneys and 22.2 per cent in the rest of the rat tissues.

The distribution of the given  $14^{14}\text{C}$  spermidine was essentially similar after subcutaneous injection. Both liver and intestine contained about 23 per cent each of the injected dose 12 hours after this administration. The fairly similar distribution of the exogenous  $^{14}\text{C}$  spermidine in the intestine and liver both after intraperitoneal and subcutaneous injections gives reason to believe that this compound is distributed through the blood circulation into the intraperitoneal organs.

In a comparable study with  $14^{14}\text{C}$  spermine  $10\ \mu\text{C}$  (about  $10^6$  cpm) was injected to rats 12 hours after intraperitoneal injection. The radioactivity was analysed in the same tissues. Table III shows that somewhat

*Table III Distribution of injected  $14^{14}\text{C}$  spermine in tissues. A comparable study to that presented in Table II but using spermine*

Tissue	Distribution of $14^{14}\text{C}$ -spermine	
	cpm per organ	per cent of dose
Liver	30,000 (285,000 — 330,000)	28.0
Kidneys	60,500 (53,800 — 64,200)	5.6
Intestine	68,000 (248,000 — 293,000)	24.8
Rest of tissues	233,000 (221,000 — 267,000)	21.6
Total	863,500	80.0

more 80.0 per cent of the given dose was recovered as unchanged  $^{14}\text{C}$  spermine in total rat. This finding suggests a slower turnover rate of exogenously administered  $14^{14}\text{C}$  spermine than  $14^{14}\text{C}$  spermidine. The distribution of the unmetabolized  $^{14}\text{C}$  spermine in the tissues differs from that of  $^{14}\text{C}$  spermidine too. In the liver (28.0 per cent) and especially in the kidneys (5.6 per cent) proportionally more was found than after injection of  $^{14}\text{C}$  spermidine.

Labelled polyamines were also administered *per os*  $0.5\ \mu\text{C}$  ( $5 \times 10^5$  cpm) in 0.5 ml of 0.9 per cent sodium chloride was given through a thin cathetre into the stomach of the rats. The radioactivity in the intestinal

*Table IV Absorption of labelled polyamines after peroral administration of 0.5  $\mu$ C (about  $5 \times 10^5$  cpm) of 1-4  $^{14}$ C labelled spermidine or spermine were administered per os After 1 to 16 hours the rats were sacrificed 3 weeks old rats were used Means of 2 to 3 animals in a group are shown Total radioactivities per organ are presented*

Time after administration in hours	After peroral administration of 1-4 $^{14}$ C spermidine				After peroral administration of 1-4 $^{14}$ C spermine			
	Radioactivity in spermidine cpm/total organ				Radioactivity in spermine cpm/total organ			
	Intestinal content	Intestinal tract	Liver	Other tissues	Intestinal content	Intestinal tract	Liver	Other tissues
1	230 000	150 000	15 000	9 800	340 000	140 000	1 000	440
3	260 000	130 000	31 000	17 000	310 000	46 000	8 000	1 000
5	330 000	73 000	19 000	16 000	140 000	24 000	1 400	1 100
8	190 000	79 000	22 000	11 000	150 000	34 000	1 400	1 100
16	3300	18 000	15 000	10 000	5 800	2 000	1 300	950



tract and its contents in the liver and the rest of the rat tissues was analysed 1 to 16 hours after a peroral treatment. During the experiment the rats were fed otherwise *ad libitum* by a standard diet. One month old rats were used. The results, as seen in Table IV, revealed that both labelled polyamines can be absorbed from the intestinal tract. The amounts of absorbed  $14^{14}\text{C}$  spermidine and  $14^{14}\text{C}$ -spermine differed a great deal from each other. Around 5 per cent of the  $^{14}\text{C}$  spermidine but only 0.4 per cent of the  $^{14}\text{C}$  spermine was recovered after a peroral administration in the liver at the maximum point 3 hours. About two-thirds of both the absorbed polyamines were found in this tissue. It is difficult to define the amounts of the label in the intestinal wall although the washed intestine (intestine and colon) contained a relatively large part of the total radioactivity. Nevertheless a part of it might represent contamination by the intestinal contents. On the other hand 10 hours after peroral treatment the label in the intestinal wall was much greater than in the intestinal content. This finding speaks in favour of a marked absorption also into the intestinal wall.

There was no radioactivity in the brains of the rat or in the fetus of pregnant rats after intraperitoneal injections of  $14^{14}\text{C}$  polyamines. In these cases the experimental periods were 1 to 8 days. However the brain and the fetal tissues contained great amounts of both polyamines. There is obviously a barrier in the placental and brain membranes because there was polyamine radioactivity e.g. in the placenta after injection of both labelled amines.

The radioactivity in the blood even soon after injection with  $14^{14}\text{C}$  labelled polyamines was low, as seen in Table V. It was determined at 15, 30, 60 and 180 min and 24 hours after the intraperitoneal injections. In this case 0.2  $\mu\text{C}$  (about  $2 \times 10^4$  cpm)  $14^{14}\text{C}$  polyamines were injected into one month old rats weighing about 80 g each. 2 ml of the blood was taken by cardiac puncture and it was immediately injected into 4 ml of 0.1 N hydrochloric acid which was analysed using the routine method. The highest values of injected polyamines in the blood were found already 5 min after injection. Thereafter the polyamine radioactivity of the blood gradually decreased being a third 180 min after injection. 24 hours later only traces of the label were detected in the blood.

*Table V* 1,4  $^{14}\text{C}$  polyamines in the blood after an intraperitoneal injection 0.2  $\mu\text{C}$  ( $2 \times 10^5$  cpm) 1,4  $^{14}\text{C}$  spermidine and spermine were injected into one month old rats. At time were analysed 2 ml of blood was taken by cardiac puncture. The results are presented as cpm/ml of blood

Time after injection	1,4 $^{14}\text{C}$ -polyamines in blood	
	After injection of 1,4 $^{14}\text{C}$ spermidine	After injection of 1,4 $^{14}\text{C}$ spermine
	Spermidine cpm/ml	Spermine cpm/ml
5 min	670	740
15 min	550	660
30 min	340	400
60 min	60	170
180 min	210	240
24 hours	traces	traces

## DISAPPEARANCE OF 1,4 $^{14}\text{C}$ POLYAMINES IN TISSUES

The elimination of the injected 1,4  $^{14}\text{C}$  polyamines will be presented as a function of time in some tissues. At appropriate time intervals after injection the radioactivity of the given polyamine has been analysed in the liver, kidneys and intestine. The first analyses in these experiments were performed 3 hours after injection. Both labelled spermidine and spermine were administered intraperitoneally. The dose of radioactivity was 0.5  $\mu\text{C}$  per 100 g rat weight, about 5 000 cpm per  $\square$ . 11/2 months old rats were used.

The elimination of 1,4  $^{14}\text{C}$  spermidine is presented in Fig. 1. The values are shown as counts per min per gram of tissue (wet weight). It is seen that the radioactivity in spermidine declines rapidly in the kidneys and in the liver during the first 24 hours after the injection. Later the decrease is gradually smaller. The behaviour of the  $^{14}\text{C}$  spermidine in the intestine is unexplained. It does not actually seem to decrease

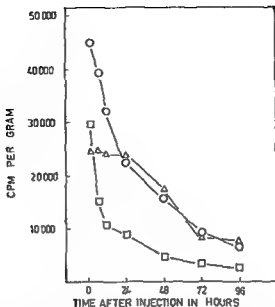


Fig 1 Disappearance of injected  $14^{14}\text{C}$  spermidine in a few tissues  
 $0.5 \mu\text{C}$  (about  $5 \times 10^5$  cpm) per 100 g rat weight of  $14^{14}\text{C}$  spermidine was injected intraperitoneally 3 to 96 hours after injection the radioactivity remaining in the spermidine was analysed. The age of the rats was one month. Mean values of 3 to 4 animals in one group are presented  
 ○ liver □ kidneys Δ intestine

during the first 24 hours after the injection. After this period the elimination is fairly similar to that in the other tissues.

In addition it can be seen from Fig 1 that the radioactivity in all the three tissues was about 6 to 10 times higher per gram of tissue at the beginning of the experiment than the dose of radioactivity was per gram of rat weight. The  $14^{14}\text{C}$  spermidine radioactivity per gram 3 hours after injection was about 46 000 in the liver, about 30 000 in the kidneys and about 24 000 cpm/g in the intestine, whereas the dose was only about 5 000 cpm/g of rat weight.

In Fig 2 the elimination of  $14^{14}\text{C}$  spermine is shown. The same tissues have been analysed. When the elimination of  $^{14}\text{C}$  spermine is compared to the corresponding elimination of  $^{14}\text{C}$  spermidine, some clear differences can be noticed. The radioactivity of spermine decreases rather slowly in the liver and kidneys. On the other hand, the elimination of  $^{14}\text{C}$  spermine seems in this case to be most rapid in the intestine. These results show that the turnover rate of exogenously administered  $14^{14}\text{C}$ -

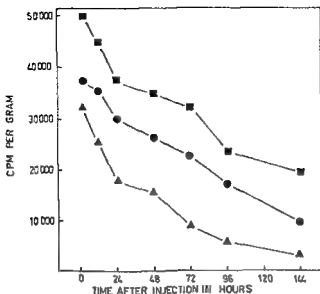


Fig 2 Disappearance of injected 14 <sup>14</sup>C spermine in a few tissues  
A comparable study to that presented in Fig 1 ● liver ■ kidneys ▲ intestine

spermine is two three times slower than that of spermidine in the liver and kidneys with rats aged 1 1/2 months

Again the radioactivity of 14 <sup>14</sup>C spermidine per gram was markedly higher in these tissues than in the average tissues. In this case the activity of <sup>14</sup>C spermine per gram was at its highest level in the kidneys and at its lowest in the intestine throughout the experimental time as seen from Fig 2

The high values of the 14 <sup>14</sup>C spermine radioactivity in the kidneys agree with the nephrotoxic effect produced with this compound as observed by TABOR and ROSENTHAL (1956)

Experiments were not performed to study precisely the elimination of 14 <sup>14</sup>C-polyamines in rat at various ages. However the evidence obtained suggests that there are no essential differences between the rates of disappearance of administered polyamines according to age. Newborn rats were the only exception. The total radioactivity in spermidine and spermine decreased markedly more slowly after injection of <sup>14</sup>C spermidine in newborn rats during the four first day of life than in older rats

## EXCRETION OF RADIOACTIVITY DERIVED FROM 14 <sup>14</sup>C-POLYAMINES

In this section the elimination of the label derived from 14 <sup>14</sup>C spermidine and 14 <sup>14</sup>C spermine into the urine and intestine as well as the degradation into carbon dioxide was studied after an intraperitoneal injection of these labelled amines. Unfortunately only the fate of two labelled carbon atoms in these molecules could be investigated. In fact the same carbon atoms were labelled both in spermidine and spermine the two at the ends of the 4 carbon chains which are derived from putrescine molecule.

### EXCRETION INTO URINE

It was expected that radioactive spermidine and spermine injected to rats would partly be excreted unchanged in the urine. In fact ROSENTHAL and TABOR (1956) have shown that a part of the administered polyamines were excreted into the urine by injecting nonisotope polyamines to rats, mice and rabbits. However they used considerably higher quantities of these substances, about 0.025–0.07 mmole per 100 g rat weight than used in this work. The present experiments were performed to measure the amounts of labelled polyamines in the urine after administration of trace amounts of <sup>14</sup>C spermidine and <sup>14</sup>C spermine.

The urine was collected daily after intraperitoneal injection of labelled polyamines. The dose of radioactivity was 0.5 to 1.0  $\mu$ C per 100 g rat weight. Comparable results were obtained with these doses. The injected radioactivity represented 0.75 to 1.49  $\mu$ moles of spermidine or 1.35 to 2.30  $\mu$ moles of spermine. The method of urine collection has been described in the methods.

The results reveal that neither labelled spermidine nor spermine was found in the urine after injection of traces of these amines. However other unidentified radioactive compounds were excreted which yielded putrescine and polyamines during the strong acid hydrolysis. The total radioactivity of the alkaline butanol soluble fraction of the urine has been demonstrated in Fig. 3 during eight days after injection. This fraction represents around 85 to 95 per cent of the total radioactivity in the urine. The urinary radioactivities after injection of labelled spermidine and sper

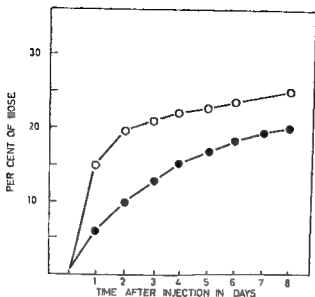


Fig 3 Excretion of radioactivity derived from injected  $14^{14}\text{C}$  polyamines into urine

20  $\mu\text{C}$  (about  $10^6$  cpm)  $14^{14}\text{C}$  labelled spermidine or spermine was injected intraperitoneally. After injection the urine was collected daily. The alkaline butanol soluble radioactivity of the urine is demonstrated as a percentage of the dose. The age of the rats was one month. The radioactivity after injection of  $14^{14}\text{C}$  spermidine  $\circ$  and  $14^{14}\text{C}$  spermine  $\bullet$ .

mine can be seen as a percentage of the daily dose in Fig 3 15 per cent of the given dose was excreted in the urine during the first day when  $^{14}\text{C}$  spermidine had been administered. After the fourth day the daily excretion represented only about one per cent of the dose. The radioactivity in the urine derived from  $^{14}\text{C}$  spermine was about 6 per cent during the first day. It seems to rise more rapidly during the 2nd - 8th day than the radioactivity derived from  $^{14}\text{C}$  spermidine.

#### EXCRETION INTO INTESTINE

Polyamines can be converted into each other in rat tissues as will be shown later. However a possibility is that the tissues themselves do not perform the interconversion of exogenously administered polyamines. To try to exclude the possibility that intestinal microorganisms might be responsible for these reactions the intestinal content was analysed after intraperitoneal injections of the label.

14 <sup>14</sup>C-polyamines were injected intraperitoneally. The analyses were made 1 to 4 days after injection. The radioactivity of the polyamines was determined from the total content of the washed intestine and colon which was diluted with 0.1 N hydrochloric acid before the routine analysis. The animals were fed *ad libitum* during this experiment.

In Table VI the total radioactivities of polyamines in the intestinal content are given when 0.5  $\mu$ C (about  $5 \times 10^5$  cpm) <sup>14</sup>C spermidine or <sup>14</sup>C spermine had been administered separately. Both labelled polyamines were discovered inside the intestine. The values represent about one per cent of the dose per day. During the second day the radioactivity of spermine increased to about double in the intestinal content after <sup>14</sup>C spermidine injection as did also the radioactivity of spermidine after a <sup>14</sup>C spermine administration.

When the slight absorption of these amines after peroral treatment (Table IV) is kept in mind it is not obvious that the small amounts of formed polyamines in the intestinal contents could practically be responsible for the interconversion in tissues.

No other alkaline butanol soluble compounds with the label could be detected in the acid intestinal content.

*Table VI* The labelled polyamines in the intestinal contents after an injection of both 14 <sup>14</sup>C spermidine and spermine. The intraperitoneal dose of radioactivity was 0.5  $\mu$ C ( $5 \times 10^5$  cpm) per 100 g rat weight. The total contents of the intestine and colon were analysed 1 to 4 days after injection. Means of 3 animals in a group are shown.

Time after injection in days	Radioactivity in intestinal content (cpm/total)			
	After injection of 14 <sup>14</sup> C spermidine		After injection of 14 <sup>14</sup> C spermine	
	Spermidine	Spermine	Spermidine	Spermine
1	7800	1500	800	6100
2	8200	3200	1600	4800
3	4000	3100	1700	3100
4	2900	2000	950	2300

## DEGRADATION TO CARBON DIOXIDE

When the recovery of the given radioactivity was studied the day after intraperitoneal injection of labelled polyamines a constant loss of radioactivity was observed which was more marked after the administration of  $^{14}\text{C}$ -spermidine. Attention was paid to the possibility that polyamines are degraded also to respiratory carbon dioxide.

1.0  $\mu\text{C}$  (about  $10^6$  cpm) 1.4  $^{14}\text{C}$  spermidine and spermine were injected intraperitoneally to the rats. One to two month old rats were used. In Table VII the mean values obtained during the first 12 hours after injection of the label are given. The amounts of  $^{14}\text{C}$  labelled carbon dioxide are expressed as a percentage of the injected radioactivity and as the total radioactivity at intervals of two hours. Accordingly a part of the labelled polyamines is degraded to respiratory carbon dioxide. About 8 per cent

*Table VII Degradation of the label derived from 1.4  $^{14}\text{C}$  polyamines to respiratory  $\text{CO}_2$*

*1.0  $\mu\text{C}$  (about  $10^6$  cpm) of 1.4  $^{14}\text{C}$  spermidine and spermine was injected intraperitoneally. The labelled  $\text{CO}_2$  was collected from the outlet air for 12 hours after injection. Means of 3 animals in a group are shown. The total radioactivities in  $\text{CO}_2$  and the radioactivities as a percentage of the injected dose are presented at time intervals of 2 hours.*

Time intervals after injection in hours	Radioactivity in carbon dioxide			
	After injection of 1.4 $^{14}\text{C}$ spermidine		After injection of 1.4 $^{14}\text{C}$ spermine	
	cpm/total $\text{CO}_2$	per cent of dose	cpm/total $\text{CO}_2$	per cent of dose
0—2	26 000	2.1	8 700	0.7
2—4	18 000	1.4	7 400	0.6
4—6	13 000	1.1	6 600	0.5
6—8	15 000	1.2	8 300	0.7
8—10	14 000	1.2	8 400	0.7
10—12	13 000	1.1	5 800	0.5
0—12	98 300	8.2	45 300	3.7



and RAINA 1966) and ornithine (RAIHÄ JANNE and SUIHKONEN 1967) are known to serve as precursors of spermidine and spermine in rat tissues. According to the evidence from these spermidine is a precursor of spermine. The present experiments were designed to confirm the synthesis of spermine from spermidine in rat tissues by the use of exogenous  $^{14}\text{C}$  labelled spermidine as a precursor.

In this study labelled spermidine was injected into rats intraperitoneally. The dose of radioactivity was  $0.5 \mu\text{C}$  ( $5 \times 10^5$  cpm) per 100 g rat weight. The specific activity of the injected material was 6.6 mC/mmol. This amount was small enough not to change the endogenous pool of polyamines essentially.

At appropriate time intervals after injection the livers were analysed three to six at a time. The radioactivity and amounts of liver polyamines were determined. Fig. 6 shows the specific activity of the formed spermine and remained spermidine (cpm/ $\mu\text{mole}$ ) as a function of time in the liver. The specific activity of spermine increased steadily for about four days after the  $^{14}\text{C}$  spermidine injection. Then it began to decrease but the fall

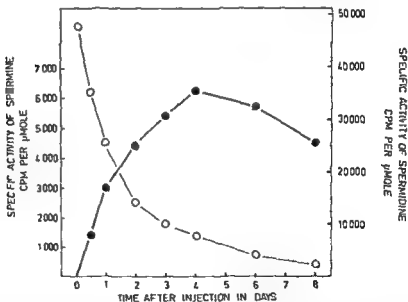


Fig. 6. Formation of spermine from  $^{14}\text{C}$  spermidine in liver.  $0.5 \mu\text{C}$  (about  $5 \times 10^5$  cpm) of  $^{14}\text{C}$  spermidine per 100 g rat weight was injected intraperitoneally. The age of the rats was one month. Mean values of 3 to 6 animals in a group are demonstrated. The specific activity of spermidine and spermine (cpm/ $\mu\text{mole}$ ) are presented as a function of time. ● spermine ○ spermidine.

was fairly slow 8 days after the spermidine injection the spermine radioactivity was still at the level in the liver that it had been at 6 days earlier. The radioactivity in the spermine exceeded that remaining in the spermidine in the liver only 8 days after the injection of labelled spermidine. The changes in the polyamine contents during these 8 days due to age variations observed by JÄNNE RAINA and SIIMES (1964) were without any practical significance in this experiment.

The spermine was synthesized to an equal degree in the liver when  $14\text{ }^{14}\text{C}$ -spermidine had been injected subcutaneously.

Only poor incorporation into spermine was noticed *in vitro* with liver slices or liver homogenate.

The methods of identifying the formed radioactivity of the electrophoretic fraction of spermine has been previously described in the methods.

### INCORPORATION OF $14\text{ }^{14}\text{C}$ SPERMINE INTO SPERMIDINE IN LIVER

The incorporation of radioactivity from labelled spermidine into spermine was expected and had already been previously demonstrated by different precursors in the rat. The converse case that administered  $14\text{ }^{14}\text{C}$  spermine led to the formation of spermidine was a somewhat unexpected

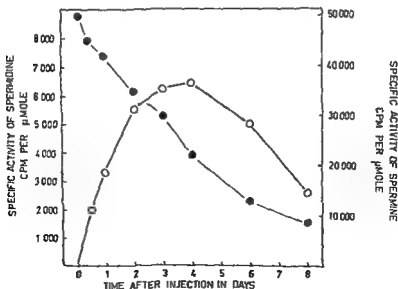


Fig 7 Formation of spermidine from  $14\text{ }^{14}\text{C}$  spermine in liver  
A comparable study to that represented in Fig 6 ○ spermidine ● spermine

observation in rat tissues. In these experiments 0.5  $\mu\text{C}$  (about  $5 \times 10^5$  cpm)  $^{14}\text{C}$  labelled spermine (4.2  $\mu\text{C}$   $\mu\text{mole}$ ) per 100 g rat weight was injected intraperitoneally.

First the incorporation into spermidine was studied at appropriate time intervals in the liver after an injection of labelled spermine. Fig. 7 demonstrates the specific activity of spermidine and spermine as a function of time. The rate of degradation of  $^{14}\text{C}$  spermine to spermidine through the loss of a propylamine group is seen to be linear during the first two days after injection. The maximum point in this reaction occurs at 3 to 4 days. The specific activity of spermidine 8 days after the  $^{14}\text{C}$ -spermine administration was about half of the formed spermine in the converse reaction seen in Fig. 6. This is the only essential difference when compared to the corresponding curve of the spermine formation. These observations also confirm that the turnover rate of spermidine formed in cells is about twice as high as that of spermine.

The experiments demonstrated in Fig. 6 and 7 show that the reactions between exogenously administered polyamines are reversible in the rat liver.

**Table VIII** Formation of spermine from  $^{14}\text{C}$  spermidine in tissues at different ages 48 hours after injection with labelled spermidine.

The analyses were performed on newborn and 3, 5, 8 and 30 week old rats. The dose of radioactivity was 0.5  $\mu\text{C}$  per 100 g rat weight (about  $5 \times 10^5$  cpm per g rat weight) in every age group. Means of 3 to 4 animals in a group are shown except the newborn values which are means of 3 pooled samples. The values have been presented as counts per min per gram of tissue (wet weight).

Age of rats in weeks	Formation of spermine (cpm/g)			
	Liver	Kidneys	Pancreas	Intestine
Newborns	1 400	3 000	—	1 900
3	1 600	2 200	1 300	2 500
5	2 900	3 000	2 400	4 600
8	4 100	2 600	2 300	4 300
30	3 800	4 600	2 000	5 800

Here also only poor incorporation was observed *in vitro* with liver slices or liver homogenate

The purification and identification experiments of the formed spermidine radioactivity have been described in the methods

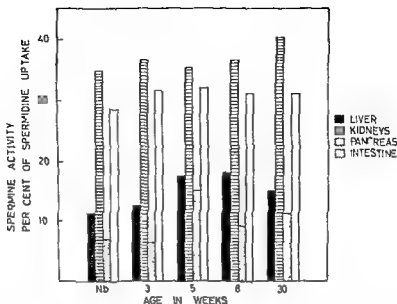
#### INTERCONVERSION OF 14 <sup>14</sup>C-POLYAMINES IN DIFFERENT TISSUES IN RELATION TO AGE

The polyamine contents in various rat tissues differ considerably from each other. The molar ratio spermidine/spermine is unusually high in the rat pancreas and rather low in the kidneys (ROSENTHAL and TABOR 1956). In addition it has been observed that the concentration of spermidine decreases clearly with age per gram of tissue (wet weight) in the rat tissues. The fall is greatest during the first month of life. Because the changes in spermine contents are generally small the molar ratio of spermidine to spermine decreases in relation to age in every tissue studied (JÄNNE RAINA and SIIMES 1964).

In this section the appearance of radioactivity in spermidine and spermine after injection of 14 <sup>14</sup>C labelled polyamines is compared with age. The liver, kidneys, pancreas and intestine were analysed. The experiments were carried out with newborn rats and 3, 5, 8 and 30 weeks old rats. In this case the analyses were performed only 48 hours after an intraperitoneal injection of labelled spermidine or spermine. The dose of radioactivity was 0.5  $\mu$ C (about  $5 \times 10^5$  cpm) per 100 g rat weight in every age group.

Table VIII represents an experiment showing the synthesis of spermine from <sup>14</sup>C spermidine in the liver, kidneys, pancreas and intestine. The values are expressed as counts per min per gram of tissue (wet weight). It is difficult to compare the spermine synthesis in these tissues without simultaneously paying attention to the corresponding uptake of the spermidine in these tissues. Accordingly Fig. 8 shows the radioactivity in spermine as a percentage of the spermidine uptake 48 hours after injection of 14 <sup>14</sup>C spermidine. It can be seen that the variation with age in the spermine synthesis from labelled spermidine is fairly small in every tissue studied. Actually there are quantitative changes in the radioactivity of

\*) In this study the uptake of a polyamine is defined as the total radioactivity which can be found in a tissue at a certain time. In this case 48 hours after a <sup>14</sup>C polyamine injection. From practical point of view the uptake of one of the polyamines is the total radioactivity in spermidine and spermine because no other radioactive compounds were detected in the rat tissues as demonstrated in the methods.



**Fig 8** Percentage formation of spermine from  $1.4^{14}\text{C}$  spermidine in a few tissues in relation to age  
Spermine radioactivity has been shown as a percentage of the spermidine uptake (radioactivity in spermidine and spermine 48 hours after injection of  $1.4^{14}\text{C}$  spermidine) The details in Table VIII

spermine with age as seen in Table VIII but they are in proportion to the corresponding uptake of spermidine in these age groups as shown in Fig 8 The spermine synthesis as a percentage of the spermidine uptake

**Table IX** Concentrations and molar ratios of polyamines in a few tissues  
The age of the rats was 30 weeks Means and ranges of 8 animals in a group are shown

Tissue	Concentrations of polyamines		Molar ratio of spermidine to spermine
	Spermidine $\mu\text{moles/g}$	Spermine $\mu\text{moles/g}$	
Liver	580 ( 545 615)	780 (742—836)	0.74 (0.69—0.82)
Kidneys	208 ( 198 214)	604 (583—638)	0.34 (0.32—0.35)
Pancreas	190 (1680 2050)	446 (417—466)	4.32 (4.72—4.00)
Intestine	760 ( 712 799)	540 (488—561)	1.40 (1.26—1.72)

is very similar throughout life especially in the kidneys and intestine. However proportionally less spermine was formed in the liver from spermidine during the first two days of life than in the livers of animals aged 5 weeks. In the present experiments the spermine concentration per liver gram (wet weight) was about twice as high as at the age of 5 weeks than newborn.

Table IX represents the concentrations and molar ratios of spermidine and spermine in some tissues in rats at 30 weeks of age. The polyamine concentrations of the liver and kidneys in relation to age were essentially similar to those reported previously by JÄNNE RAINA and SJIMES (1964). In the present study a similar decrease with age in the spermidine concentration was noticed in the pancreas as in other tissues. The behaviour of the spermidine concentration of the intestine was different per gram from other analysed tissues with age. In this organ there was practically no decrease in the spermidine concentration or in the molar ratio of spermidine to spermine in relation to age.

Fig. 8 illustrates also that in the age group of 30 weeks old rats around 40 per cent of the spermidine uptake in the kidneys and only 10 per cent in the pancreas was found as spermine 2 days after the  $14^{14}\text{C}$ -spermidine injection. The corresponding value in the liver was 15 per cent. It is interesting to compare these values with the endogenous concentrations and with the molar ratios of the polyamines in these tissues. The molar ratio of spermidine to spermine was as given in Table IX 4.3 in the pancreas and only 0.3 in the kidneys. In the kidneys proportionally

*Table X Formation of spermidine from  $14^{14}\text{C}$  spermine in tissues at different ages 48 hours after injection with labelled spermine. The comparable details are shown in Table VIII.*

Age of rats in weeks	Formation of spermidine (cpm/g)			
	Liver	Kidneys	Pancreas	Intestine
Newborns	200	1700	—	900
3	5300	4500	5400	2000
5	5100	3500	5200	3200
8	4200	3000	3800	2700
30	5000	4300	3200	3600

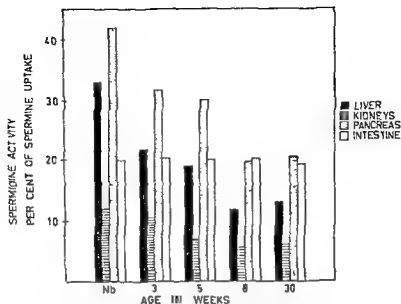


Fig ■ Percentage formation of spermidine from  $14^{14}\text{C}$  spermine in a few tissues in relation to age

Spermidine radioactivity has been shown as a percentage of the spermine uptake (radioactivity in spermine and spermidine 48 hours after injection  $14^{14}\text{C}$  spermine) The details in Table X

more spermidine was converted to spermine than in the pancreas The behaviour of the spermine synthesis in the intestine differed from the three other analysed tissues There was no similar correlation to the molar ratio of spermidine and spermine

Identical experiments were performed to study the converse reaction the incorporation of  $14^{14}\text{C}$  spermine into spermidine in relation to age Table X shows the radioactivity of spermidine per gram of tissue (wet weight) The formed radioactivity in spermidine per gram of liver is clearly higher in the young rats than the corresponding radioactivity in the spermine derived from spermidine which was shown in Table VIII On the other hand these liver values are similar when compared to each other in the older rats aged 8 or 30 weeks Analogous differences in the formation of spermidine and spermine can also be noticed in the kidneys and in the pancreas but not in the intestine

In Fig ■ the formed spermidine radioactivity is seen as a percentage of the spermine uptake This figure also shows that the conversion to spermidine is dependent on age The percentage values in young rats particularly newborn rats and those at 3 or 5 weeks of age are remark

ably higher in the liver pancreas and kidneys than those found in adult ones. The incorporation into spermidine is also here similar in this case in the age groups of eight week and thirty week old rats.

When the formation of spermidine in Fig. 9 is compared in the tissues of adult rats some correlation to the molar ratio of spermidine to spermine can here be noticed also. In the pancreas with a high molar ratio around 20 per cent and in the kidneys where the molar ratio is low, only 6 per cent of the spermine uptake is recovered in the spermidine during a period of 48 hours after the injection of  $14^{14}\text{C}$  spermine. The formation of spermidine in the intestine is very close to 20 per cent of the spermine uptake at every point of observation with age.

### INCORPORATION STUDIES WITH $14^{14}\text{C}$ POLYAMINES DURING LIVER REGENERATION

The formation of spermidine from some of its labelled precursors has been observed to be stimulated during liver regeneration. The rise in the incorporation of  $2^{14}\text{C}$  methionine into spermidine is about 10 fold during an incorporation period of 4 hours and it culminates at the point 12 to 16 hours after partial hepatectomy (RAINA, JÄNNE and SIIMES 1966). The incorporation of  $5^{14}\text{C}$  arginine (JÄNNE and RAINA 1966) and  $14^{14}\text{C}$ -ornithine (JÄNNE under preparation) is also increased into spermidine. On the other hand in a comparable study on the biosynthesis of spermidine from  $14^{14}\text{C}$  putrescine somewhat surprising results were obtained by JÄNNE and RAINA (1966). They noticed that the specific activity of spermidine was clearly decreased 6 hours after partial hepatectomy after which the spermidine values again increased gradually to about control level. In a preliminary experiment JÄNNE and RAINA (1966) also noticed that after injection of  $5^{14}\text{C}$  of  $14^{14}\text{C}$ -spermidine at a point 22 hours post-operatively the liver total spermine radioactivity was  $1 \times 10^5$  cpm one day after administration and  $3.3 \times 10^5$  cpm 5 days after administration. It was also observed that 5 days after injection the specific activity of spermine exceeded that of administered spermidine.

The concentration of spermidine per unit weight has been shown to rise markedly in the liver during regeneration. The increase in the concentration of this amine culminates at 64 to 72 hours after a partial hepatectomy (DYKSTRA and HERBST 1965, RAINA, JÄNNE and SIIMES 1965, 1966). On the other hand the spermine concentration per liver gram decreases first and reaches its lowest values 32 hours afterwards where



after the concentration of spermine gradually increases but exceeds the control level only at a point 216 hours after operation

The present experiments were designed to find some further explanation for the behaviour of polyamines and for the role of interconversion reactions between exogenously administered spermidine and spermine during the regeneration

The technique used for partial hepatectomy has been given in the methods. The operation was performed 4 to 72 hours before decapitation. The rats were injected at different times after partial hepatectomy and they were sacrificed simultaneously three hours after injection of the label. It was necessary to find as short an incorporation time as possible to reflect the quick changes in the metabolism of polyamines after partial hepatectomy. Incorporation times of one to 24 hours (SIIMES 1966) were tested but that of three hours was selected for these experiments. During that time the changes in the radioactivities were large enough for reliable measurements. Three hours was also supposed to be a time short enough

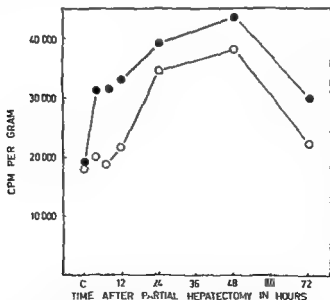


Fig 10 Uptake of  $^{14}\text{C}$  polyamines in regenerating liver

Partial hepatectomy was performed 4 to 72 hours before decapitation. Two separate experiments are presented: one in which  $0.2 \mu\text{C}$  (about  $2 \times 10^5$  cpm)  $^{14}\text{C}$  spermidine and another in which  $0.2 \mu\text{C}$  (about  $2 \times 10^5$  cpm)  $^{14}\text{C}$  spermine was injected intraperitoneally 3 hours before analysis. Mean values of 4 animals in a group are presented. The age of the rats was one month.

○ spermidine ● spermine

to reflect the changes in the polyamine metabolism. The incorporation of radioactivity during this 3 hour period before decapitation was used as a measure of the degree of stimulation of the polyamine conversion. 0.2 to 0.5  $\mu$ C 14  $^{14}$ C labelled spermidine and spermine were separately injected intraperitoneally. One-month-old rats were used.

**Uptake of administered 14  $^{14}$ C-polyamines** First in Fig. 10 the uptake of injected 14  $^{14}$ C spermidine and 14  $^{14}$ C spermine are seen in the regenerating liver 3 hours after injection. The values are expressed as counts per min per gram of liver (wet weight). The uptake of  $^{14}$ C spermine begins to rise soon after partial hepatectomy. It is already nearly double 4 hours after the operation compared to the untreated controls. The maximum point of spermine uptake is reached 48 hours after operation.

The curve presenting the uptake of 14  $^{14}$ C spermidine in Fig. 10 differs to some extent from that of 14  $^{14}$ C spermine. There are no marked changes in the spermidine uptake at the time interval from 4 to 12 hours after partial hepatectomy. At 24 and 48 hours the spermidine uptake per gram of liver is clearly increased.

Fig. 11 represents two separate experiments, one in which the incorporation of radioactivity from 14  $^{14}$ C spermine into spermidine is demonstrated and another in which the incorporation of 14  $^{14}$ C spermidine both into spermine and into putrescine is shown during liver regeneration.

**Incorporation of 14  $^{14}$ C spermidine into spermine** In this reaction only some slight changes seem to occur during the early period of regeneration at 4 to 12 hours after partial hepatectomy as seen in Fig. 11. At the later stages of the observation, i.e. 24 and 48 hours, there is a slight increase in the radioactivity of spermidine per gram culminating at 48 hours. The changes in the uptake of  $^{14}$ C spermidine (Fig. 10) and in the synthesis of spermine from exogenous  $^{14}$ C spermidine (Fig. 11) are fairly similar. Accordingly approximately equal parts of the uptaken radioactivity in the liver has been converted into spermine at every point of observation.

**Incorporation of 14  $^{14}$ C spermine into spermidine** As seen in Fig. 11 a marked stimulation is seen in this reaction during regeneration. The activity of hepatic spermidine increased from the control value 720 cpm/g to 2700 cpm/g at a point 8 hours after partial hepatectomy. Thereafter the spermidine radioactivity gradually decreased but the values still remained at a high level during the first 24 hours after

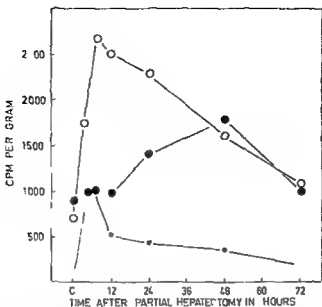


Fig. 11 Formation of spermine ● and putrescine ● from 2,4  $^{14}\text{C}$  spermidine and spermidine ○ from 2,4  $^{14}\text{C}$  spermine in regenerating liver. Partial hepatectomy was performed 4 to 72 hours before analysis. 0.5  $\mu\text{C}$  (about  $5 \times 10^5$  cpm) of 2,4  $^{14}\text{C}$  labelled spermidine or spermine was injected intraperitoneally 3 hours before decapitation. The age of the rats was one month. Mean values of 4 animals in a group Cpm per gram of liver (wet weight) demonstrated.

operation. The rise in the incorporation into spermidine seems to be quantitatively the most remarkable change of all the reactions studied during the liver regeneration. In addition the specific activity of spermidine increased: at 4 hours it was about 2.5 times and at 8 hours about 4 times the control values. At the latest stages of observations the specific activity of the formed spermidine gradually decreased below the control values.

**Incorporation of  $^{14}\text{C}$  spermidine into putrescine.** It was surprising to notice that there was some radioactivity in the putrescine fraction after administration of  $^{14}\text{C}$ -spermidine. However by the standard method the radioactivity in the putrescine barely exceeded that of the background. When the final concentration of the liver samples was 8-fold that of the routine one there was enough radioactivity that it could just be measured in the untreated livers. The injection of labelled spermidine soon after partial hepatectomy caused a prompt increase in the radioactivity of putrescine. At 48 hours it was considerably increased, as revealed in Fig. 11.

An identical increase was obtained from 2 to 4 hours postoperatively when the incorporation time was only one hour. The values at 2 hours were thus about half those at 4 hours. As seen in Fig. 11 the putrescine radioactivity was still increasing during the period from 4 to 8 hours. The maximum values around 8 to 10 times the controls were found 8 hours after partial hepatectomy. At an early moment after operation the concentration of putrescine per gram was increased about 2 to 3 times in the liver. There was thus a smaller rise in the specific activity of the formed putrescine during the regeneration. The experiments on the identification of the formed radioactivity in the putrescine fraction have been described in the methods.

These experiments reveal that the conversion of exogenous spermidine to putrescine is possible in the rat liver. This is worthy of note when the metabolism of polyamines is being discussed.

**Incorporation of  $14^{14}\text{C}$ -spermine into putrescine**  
No radioactivity could be demonstrated in the putrescine fraction either in the control or hepatectomized liver samples after injection of  $14^{14}\text{C}$  spermine during the regeneration.

**Incorporation experiments in vitro**  
The studies on the interconversion of labelled polyamines in vitro with liver homogenate obtained from the livers 16 and 48 hours after partial hepatectomy were not successful.

**Concentrations of polyamines**  
The changes in the concentrations of polyamines in the regenerating rat liver in the present study were comparable to those which have been reported previously by RAINA JÄNNE and SIIMES (1965, 1966).

#### INTERCONVERSION OF $14^{14}\text{C}$ POLYAMINES DURING ETHIONINE TREATMENT

Methionine through the formation of S-adenosylmethionine is a source of the three carbon moieties of spermidine and spermine in polyamine biosynthesis. After administration of ethionine to rats large amounts of S-adenosylethionine are found in the liver (MODY, BULBA, HOLO, WECKY and STEKOL 1963; RAINA JÄNNE and SIIMES 1964; SHULL, McCONOMY, VOGT, CASTILLO and FARBER 1966). This compound is obviously unavailable for polyamine synthesis although no experimental evidence supports this conclusion. Although many changes in tissue metabolism arise from ethionine administration most of them inhibitory to protein synthesis (STEKOL 1963) this compound also causes characteristic

changes in the polyamine concentrations during continuous daily administration over several days first a decrease later a considerable rise in the spermidine and a fall in the spermine concentration per gram of rat liver (wet weight) (RAINA JÄNNE and SIIMES 1964 KREZNER and STARR 1966) After a single injection of ethionine variable changes in the polyamine concentrations have been reported in the rat liver (RAINA JÄNNE and SIIMES 1964 SHULL McCONOMY, VOGT CASTILLO and FARBER 1966 NEISCH 1967)

In the following experiments DL ethionine was injected daily intraperitoneally for six days before decapitation The dose of ethionine was 0.25 or 0.6 mmole per 100 g rat weight per day A single dose of 1.4  $^{14}\text{C}$  spermidine 0.5  $\mu\text{C}$  ( $5 \times 10^5$  cpm) per 100 g rat weight was also injected intraperitoneally 1 to 4 days before decapitation One-month old rats were used

It can be seen from Fig. 12 that the radioactivity left in spermidine was slightly although insignificantly higher during ethionine administration compared to the controls Ethionine had practically no effect on the rate of elimination of the injected  $^{14}\text{C}$  spermidine in the liver The degradation products of spermidine during ethionine treatment are not known Only the pathway to putrescine was studied but it was not activated

In Fig. 12 the incorporation of radioactivity into spermine as a function of time in the livers of the control and ethionine treated groups are shown too The counts per total liver are presented 1 to 4 days after injection with 1.4  $^{14}\text{C}$  spermidine In this experiment the dose of ethionine was 0.25 mmole per 100 g per day but essentially comparable results were obtained during the daily dosage of 0.6 mmole Fig. 12 shows that a clear inhibition is induced in the spermine synthesis from 1.4  $^{14}\text{C}$  spermidine by daily ethionine administrations

It takes about two days before the inhibitory effect on the spermine synthesis is fully developed When the ethionine treatment was interrupted two days before decapitation under otherwise identical experimental conditions as shown in Fig. 12 the spermine synthesis resumed to some extent 1 to 3 days after interruption

A comparable study on the formation of spermidine from 1.4  $^{14}\text{C}$  spermine during ethionine treatment was also performed Daily ethionine injections 0.3 mmole per 100 g rat weight per

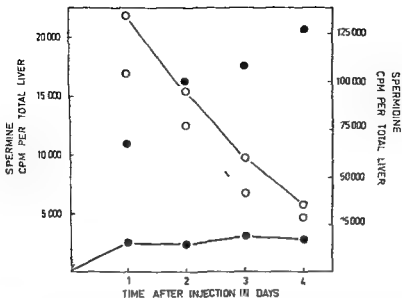


Fig 1. Effect of ethionine treatment on the elimination of injected  $1,4$   $^{14}\text{C}$  spermidine and on the synthesis of spermine in liver

DL ethionine ( $0.5$  mmole/100 g rat weight) was injected intraperitoneally and daily for 6 days before decapitation. A single injection of  $1,4$   $^{14}\text{C}$  spermidine ( $0.5$   $\mu\text{C}$  about  $5 \times 10^5$  cpm) was also injected intraperitoneally 1 to 4 days before decapitation. The age of the rats was one month. Mean values of 4 animals in a group are presented. The radioactivity in spermidine and spermine per total liver is shown.  $\circ$  spermidine  $\bullet$  spermine — ethionine treated — — — controls

day had no effect on the appearance of radioactivity in spermidine in the liver when compared with the untreated livers.

### CHANGES IN LIVER POLYAMINES DURING STARVATION

During the ethionine treatment the weight of the rats fell considerably depending on the dose of ethionine. The rats were fed *ad libitum* but their food intake was quite small. To eliminate the effects of starvation as a cause for polyamine changes during ethionine administration some experiments were carried out on rats fed only with water for 1 to 4 days. Rats aged one month were used.

The concentrations of polyamines per gram liver and per total liver (wet weight) are shown in Fig 13. The spermidine concentration of the liver gram began to fall only after a 3-day period of starvation. On the 4th day the concentration of spermidine per gram was already nearly half that of the control values. The liver spermine concentration per gram rose about 20 per cent ( $p < 0.1$ ) during the first

## DISCUSSION

**Metabolism of polyamines in the rat** Methionine putrescine arginine and ornithine (RAINA 1964 JÄNNE and RAINA 1966 JÄNNE under preparation) have been reported to be precursors of spermidine in the rat Arginine and ornithine are shown to be precursors in the biosynthesis of putrescine also in the rat liver (JÄNNE under preparation) Putrescine has also been demonstrated to be incorporated into spermidine in microorganisms (TABOR ROSENTHAL and TABOR 1956 1958) in Ehrlich ascites cells (SIIMES and JÄNNE 1967) and in the developing chick embryo (RAINA 1962 b 1963) The radioactivity in putrescine is also incorporated into spermine but much more slowly than into spermidine This slow incorporation into spermine gives cause to believe that spermidine is a precursor of spermine (RAINA 1963) Further support for this idea has been obtained by RAINA (1963) in experiments with developing chick embryo After administration of labelled methionine which is the source of the 3 carbon chains of polyamines radioactivity was incorporated simultaneously into both polyamines in roughly equivalent amounts These experiments also provided evidence that both propylamine moieties of spermine are derived from methionine On the other hand it has been pointed out that *in vitro* systems spermidine could not form a substitute for putrescine as an acceptor of the propylamine moiety (TABOR 1962 b)

The present experiments as well as the preliminary experiments from this laboratory (JÄNNE and RAINA 1966 SIIMES 1966 1967) demonstrated by use of exogenous spermidine that spermidine is a precursor of spermine in rat tissues No evidence for some other metabolic route leading to the formation of spermine other than through spermidine has been presented Evidence that spermine can be converted into spermidine has been obtained in micro-organisms (RAZIN BACHRACH and GERY 1958 RAZIN GERY and BACHRACH 1959 BACHRACH PERSKY and RAZIN 1960) Further the degradation of spermidine to putrescine has been reported in *Pseudomonas aeruginosa* (PADMANABHAN and KIM 1965) The reversible interconversion between putrescine spermidine

and spermine in a single organism has not previously observed although the reactions have been separately demonstrated in different organisms. The present work shows that exogenous spermidine and spermine can be converted into each other and that spermidine can also be degraded to putrescine in rat liver. These observations indicate that there are reversible reactions between putrescine and polyamines in the rat.

In addition to the interconversion reactions the polyamines can be metabolized through other routes in rat tissues. This area of polyamine metabolism was not studied in detail in the present work. However it was noticed that a considerable portion of radioactivity was found as  $\text{CO}_2$  derived from the carbon 1 and 4 in spermidine and spermine. The metabolic routes of this degradation are not known. The formation of carbon dioxide from 1,4- $^{14}\text{C}$  spermine was considerably smaller than that from 1,4- $^{14}\text{C}$  spermidine under the same experimental conditions. It is possible that the degradation of spermine might go through spermidine to  $\text{CO}_2$ .

Several radioactive metabolites of polyamines and putrescine were found in the alkaline butanol soluble extract of the urine during a period of 8 days after injection with 1,4- $^{14}\text{C}$  polyamine. These compounds were not identified in this study. Similar radioactive compounds were not found however in the liver 24 or 48 hours after injection. It was interesting to note that the strong acid hydrolysis of the acid urine yielded large amount of putrescine in addition to polyamines. In other experiments putrescine was found to be a degradation product of 1,4- $^{14}\text{C}$  spermidine in regenerating liver but not in the normal liver. The studies on the urine indicate that the untreated animal might to a large extent also have the ability degrade spermidine to putrescine which would be excreted into the urine as a conjugate e.g. as acetylputrescine.

The experiments with the chick embryo indicated the slow elimination of endogenous polyamines (RAINA 1963). It was also obvious that the turnover rate of spermine was slower than that of spermidine. Similar findings were also obtained in the present experiments. The elimination of both the exogenous spermine and that formed in the cells was clearly slower than that of exogenous spermidine. For instance in the liver 8 days after the 1,4- $^{14}\text{C}$  spermidine injection the amount of radioactive spermine exceeded that of spermidine. The slower degradation to  $\text{CO}_2$  and the slower excretion of radioactivity into the urine after injection with  $^{14}\text{C}$ -spermine also indicate a slower turnover rate in spermine than in spermidine.



It has been observed that *E. coli* (TABOR and TABOR 1964 a 1966) and Ehrlich ascites cells (JÄNNE and SIIMES, under preparation) can as well take up putrescine and polyamines as synthesize these compounds. Naturally the present results concerning the uptake of labelled polyamines do not define the localization of the label in the cells. According to the studies with *E. coli* and Ehrlich ascites cells it is obvious that a part of the label in the medium is adsorbed on the cells and a part is transported into the cells. It is likely that a major part of the shown uptake is intracellular as late as 48 hours after injection of a labelled polyamine.

The importance in animals between the exogenous and the polyamines synthesized in the tissues is not known. The present results revealed that part of the polyamines and particularly of the spermidine can be absorbed from the intestine after peroral treatment. Rats have also been fed on a diet which lacked polyamines (ROSENTHAL and TABOR 1956). This treatment caused only a slight decrease in the liver concentration of spermidine. A possibility might also be that the intestinal micro-organisms synthesize polyamines which are then partly absorbed from the intestine. This question has been studied with the germ free rats of a third generation by ROSENTHAL and TABOR (1956). However, no large deviations were noticed in the liver concentrations of polyamines. These observations suggest the importance of the biosynthesis of polyamines in rat tissues instead of the absorption from the intestine.

**Metabolism of polyamines during starvation**  
During starvation the liver contents of the polyamines decreased clearly that of spermidine somewhat sooner than spermine per total liver. It is likely that during starvation there is a deficiency of the essential precursor  $\alpha$ -methylmethionine needed in the biosynthesis of polyamines. In fact the concentration of S-adenosylmethionine has been reported to be nearly half of that of the normal liver value even after starvation lasting only one day (SCHLENK 1965). This might be a reason for the changes in polyamine concentrations during starvation. Because of the slower turnover rate of spermine the decrease in the concentrations would first act on the spermidine. As a result the molar ratio of spermidine to spermine would lessen during starvation. The lack of spermidine is thus more intensive than that of spermine. This would explain the increased degradation of 14  $^{14}\text{C}$  spermine to spermidine in the starved livers.

**Polyamine interconversion in relation to age**  
The varying concentrations of polyamines in different tissues and the fall in the tissue spermidine concentration with age (JÄNNE, RAINA and SIIMES 1964) are of interest. In the present study some attempts were made to study the interconversion of polyamines in relation to age. The clearest way to study these reactions in the tissues according to age would have obviously been the incorporation studies *in vitro* e.g. with liver slices or homogenate obtained from the rats of different ages. Unfortunately these amines did not incorporate into each other under such conditions although the interconversion has been demonstrated in micro organisms (TABOR and TABOR 1964b) and in Ehrlich ascites cells (SIIMES and JÄNNE 1967).

It is obvious that the interconversion between spermidine and spermine is partly responsible for the changes in polyamine concentrations with age. The results showed that proportionally more of the exogenous spermine taken up in a tissue was converted into spermidine if the molar ratio of endogenous spermidine to spermine was high. On the other hand the formation of spermine from spermidine was more active in tissues with a low molar ratio. The mode of expressing the formation of polyamines from each other used here is questionable in the tissues of new born rats because the elimination of the exogenous spermidine given was slower in the tissues at least during the first four days of life. However the rate of elimination of exogenous spermidine in the 3-30 week age group and that of exogenous spermine in every age group was found to be essentially similar in the tissues analysed. In young rats aged 3 to 5 weeks more spermidine was clearly formed from exogenous spermine than spermine from exogenous spermidine during the 48 hours after injection of the label from an equal dose of a given polyamine. This was true in the liver, kidneys and pancreas but not in the intestine. In addition when the whole animal was analysed more spermidine was formed in those age groups. In the tissues mentioned there were practically no difference in the polyamine formation among the rats aged 8 to 30 weeks. These results suggest that the formation of spermidine from labelled spermine is more active in young rats than in older ones.

The above mentioned findings prompted a study of the metabolism of polyamines in a rapidly growing system which might give more quantitative data than the experiments relying on growth with age. Regenerating rat liver was chosen for these experiments.

**Polyamine metabolism in regenerating liver and relation to nucleic acids** The blood flow through the two liver lobes remaining after partial hepatectomy  $\equiv$  increased during the 3 first hours after operation (MENYHART and SIMON 1966) LIEBERMAN, KANE and SHORT (1965) reported an interesting study in which they had experimentally investigated the effect of portal vein volume on the synthesis of RNA in the liver They noticed that rapid portal injections of a variety of solutions e.g. sodium chloride caused increases in the specific activities of RNA-polymerase and in the rates of the RNA synthesis These rises were indistinguishable from those obtained after partial hepatectomy Several changes in the formation of putrescine, spermidine and spermine were noticed in this work during the liver regeneration It is difficult to exclude the possibility that these changes would be partly the result of the increased blood flow through the regenerating lobes of the liver On the other hand the concentrations of radioactive polyamines in the blood were fairly low even soon after the intraperitoneal injections but nothing is known about the effectiveness of the liver cells in clearing polyamines from the blood If the clearance is intensive the increased blood flow obviously also increases the uptake of labelled polyamines during liver regeneration

Putrescine synthesis from its labelled exogenous precursors such as 5- $^{14}\text{C}$ -ornithine and 5- $^{14}\text{C}$  arginine has been shown to have risen even 2 hours after partial hepatectomy and reached its highest values at around 8 hours (RÄIHÄ JÄNNE and SUIHKONEN JÄNNE under preparation) The conversion of exogenous spermidine to putrescine is activated 2 to 6 hours after partial hepatectomy as shown in the present work However during the early stage of regeneration at 6 hours the incorporation of radioactivity from exogenous putrescine into spermidine was decreased according to JÄNNE and RAINA (1966) Earlier DYKSTRA and HERBST (1965) noticed an increase in the incorporation of exogenous putrescine into spermidine but these two studies are obviously not comparable to each other There were essential differences between these experiments e.g. in the route of administration of the label The maximum concentration of putrescine can be found at around 4 to 8 hours after operation At that time it is about 3 fold that of the controls The mentioned changes in the metabolism of putrescine  $\equiv$  its increased synthesis from 5- $^{14}\text{C}$ -ornithine and arginine as well as the increased formation from 1- $^{14}\text{C}$ -spermidine and its possibly decreased incorporation into spermidine provide a good explanation for its accumulation during the early stage of liver regeneration

The synthesis of spermidine is also increased after partial hepatectomy culminating at 8 to 20 hours after operation. This is seen through the incorporation of radioactivity into spermidine from labelled methionine (RAINA JÄNNE and SIIMES 1965, 1966) arginine (JÄNNE and RAINA 1966) and spermine. The rise in spermidine radioactivity derived from exogenous spermine obviously begins immediately after partial hepatectomy and the highest values are seen at 8 hours. The formation of spermidine from  $14\text{ }^1\text{C}$  spermine remained at a high level during the first 24 hours after operation. The incorporation of radioactivity from  $14\text{ }^1\text{C}$  methionine into spermidine also rose immediately and culminated at 12 to 16 hours after partial hepatectomy (RAINA JÄNNE and SIIMES 1965, 1966). In addition the incorporation of exogenous putrescine into spermidine which was reported to have decreased at 6 hours, rose to the control level during the interval from 6 to 16 hours after operation (JÄNNE and RAINA 1966). Accordingly the stimulation in the spermidine synthesis seems to occur particularly during the first 24 hours after partial hepatectomy. As recently reported the liver spermidine concentration per weight unit begins to rise at a point 16 hours after operation. It reaches its highest values at 64 to 72 hours (DYKSTRA and HERBST 1965, RAINA JÄNNE and SIIMES 1965, 1966).

A lag period during the first 10 days of life has been noticed in the rat liver during the postnatal growth. During this period liver weight does not increase (OLIVER BALLERD SHIELD and BENTLEY 1962). The liver of 3 week old animals grows rapidly and might be comparable to a regenerating liver. In age groups of 3 to 5 weeks the formation of spermidine from exogenous spermine was the prominent feature in this reversible reaction as was also shown during the liver regeneration.

In contrast no noteworthy changes could be found in the spermine synthesis from exogenous spermidine at 4 to 12 hours after partial hepatectomy. At that time there was practically no stimulation either in the incorporation into spermine from exogenous methionine (RAINA JÄNNE and SIIMES 1965, 1966) although in the untreated livers this precursor was incorporated into both polyamines in roughly equivalent amounts (RAINA 1964). In the light of the above findings the activated degradation of exogenous spermine to spermidine at the early stage of regeneration might lead to a fall in the concentration of spermine per gram of liver (wet weight) seen during the period of 16 to 32 hours (RAINA JÄNNE and SIIMES 1965, 1966). The rise in the incorporation of radioactivity from  $14\text{ }^1\text{C}$  spermidine to spermine was found to occur at a fairly late stage.

after operation : ■ at 24 and 48 hours after partial hepatectomy The total contents of spermine per residual liver is slowly increasing in the regenerating liver As late as 96 to 216 hours after operation the spermine contents have clearly exceeded the control level (RAINA JÄNNE and SIIMES 1966)

A phasic increase in the concentrations of putrescine spermidine and spermine is obvious during liver regeneration The rise in liver putrescine culminates at 4 to 8 hours and that of spermidine around 64 to 72 hours and the concentration of spermine is still slowly increasing 216 hours after partial hepatectomy On the other hand the concentrations of most free amino acids and related compounds in the liver stay unchanged at 6 and 24 hours postoperatively (FERRARI and HARKNESS 1954) except those of aspartic acid glutamic acid and lysine which have been shown to be clearly elevated 6 hours after partial hepatectomy (FERRARI and HARKNESS 1954) A less marked increase in the above-mentioned amino acids was noticed at 24 hours The concentration of glutamine has been reported to be decreased in regenerating rat liver (CHRISTENSEN, ROTH WELL SEARS and STREICHER 1948 FERRARI and HARKNESS 1954)

During the last few years evidence has been accumulated on the relation of polyamines to nucleic acids A very close correlation between the concentrations of total polyamine nitrogen and RNA phosphate has been observed during the exponential growth of *E. coli* by RAINA and COHEN (1966) and by COHEN HOFFNER JANSEN MOORE and RAINA (1967) These correlations are interesting because it has been noticed a stimulatory effect by polyamines and putrescine on DNA primed RNA polymerase activity *in vitro* (KRAKOW 1963, FOX ROBINSON, HASELKORN and WEISS 1964 FOX and WEISS 1964) and on the DNA polymerase (BREWER and RUSCH 1966) In a polyauxotrophic strain of *E. coli* RAINA and COHEN (1966) reported that a relatively high concentration of exogenous spermidine caused a marked rise in the uracil incorporation into RNA during amino acid starvation An increase in RNA synthesis has also been obtained by spermine in *E. coli* (MILLS and DUBIN 1966) and in a tissue culture (GOLDSTEIN 1965)

In animal tissues correlations between the changes in the concentrations of nucleic acids and polyamines particularly between those of RNA and spermidine have also been noticed e.g. during the embryonic development (RAINA 1963 CALDARERA BARBIROLI and MORUZZI 1965) in the rat liver during ionizing radiation (CALDARERA COZZANI and MO

RUZZI 1966) and during growth hormone treatment (JÄNNE 1967) and especially in the intracellular distribution of RNA and spermidine (RAINA and TELARANTA 1967)

The changes in the concentrations of RNA and spermidine have been shown to be fairly similar during liver regeneration (DYKSTRA and HERBST 1965 RAINA JÄNNE and SIIMES 1965 1966) On the other hand a few hours after partial hepatectomy there is a rise in the synthesis of RNA reflected in the increased incorporation of labelled orotic acid (HECHT and POTTER 1958 FUJIOKA KOGA and LIEBERMAN 1963 UCHIYAMA FAUSTO and LANCKER 1966) orthophosphate (NYGAARD and RUSCH 1955 WELLING and COHEN 1960) and glucose (SCHNEIDER and POTTER 1957) into RNA The RNA polymerase activity has also been reported to increased in the liver after partial hepatectomy A certain rise has been noticed even at 2 to 6 hours The maximum values have been found 12 to 24 hours after operation (BUSCH CHAMBON MANDEL and WEILL 1962 TSUKADA and LIEBERMAN 1964 RO and BUSCH 1967) In contrast a rise in the incorporation into DNA occurs later 15 to 18 hours after partial hepatectomy (NYGAARD and RUSCH 1955 HECHT and POTTER 1956 1958 SCHNEIDER and POTTER 1957 WELLING and COHEN 1960)

It is obvious that many changes occur in the metabolism of polyamines and putrescine before the tissue actually begins to grow In the regenerating liver the formation of putrescine and spermidine are active at the same time as the stimulation in the synthesis of RNA

**Polyamine metabolism in relation to ethionine treatment** Ethionine is a toxic compound whose effects on tissues are obviously based on the antagonism of methionine (STEKOL 1963) During ethionine treatment relatively large amounts of S adenosylethionine are found in the rat liver (MODY BULBA HOLOWECKY and STEKOL 1963 RAINA JÄNNE and SIIMES 1964 SHULL MCCONOMY VOGT CASTILLO and FARBER 1966) The formed compound is obviously inactive as a substrate for the biosynthesis of polyamines for which S adenosyl methionine is an essential precursor (TABOR and TABOR 1964 b)

The effect of ethionine is dependent upon the daily dose the cessation of treatment and the tissue which has been analysed in the rat (SIIMES RAINA and JÄNNE under preparation) The day after a single injection of ethionine variable effects have been reported on the concentration of spermidine 0.1 mmole has caused a rise (NEISCH 1967) and 0.6 mmole

a fall (RAINA JÄNNE and SIIMES 1964 KREMZNER and STARR 1966) in the concentration of spermidine in the rat liver. With 0.2 mmole of ethionine no effect was obtained on the spermidine concentration (RAINA JÄNNE and SIIMES 1964). On the other hand during daily ethionine treatment (0.6 mmole/100 g rat weight/day) there is first a fall and later a considerable rise in the concentration of spermidine (RAINA JÄNNE and SIIMES 1964 KREMZNER and STARR 1966) and a marked fall in the concentration of spermine of the rat liver (RAINA JÄNNE and SIIMES 1964). As a consequence of these changes the molar ratio of spermidine to spermine rose markedly in the liver.

RAINA (1963) in the developing chick embryo showed that the rate of the synthesis of spermidine from  $^{14}\text{C}$  methionine was not altered by a single injection of ethionine but that this treatment caused a decrease in the incorporation of  $^{14}\text{C}$  methionine into spermine. In the present experiments a single injection of ethionine produced only a slight decrease in the incorporation of exogenous spermidine into spermine in the rat liver. However by the daily administrations of ethionine nearly the total inhibition originated in spermine synthesis from 1.4  $^{14}\text{C}$  spermidine.

An explanation for the rise in spermidine and the fall in spermine concentrations during continuous treatment of ethionine might be the inhibition in the formation of spermine from spermidine. The rise in spermidine might also be due to an increase in its synthesis. As shown in the present experiments the formation of spermidine from exogenous spermine was not altered under these conditions. JÄNNE (under preparation) has found that the synthesis of spermidine from exogenous putrescine is not increased during ethionine administration either.

During the administration of ethionine the rats are practically starved because the intake of food is very poor during this treatment. During the starvation without ethionine converse changes took place in the liver polyamine concentrations to those during ethionine treatment. These findings suggest that relative starvation during administration of ethionine is not the only reason at least for the changes in the metabolism of polyamines.

## SUMMARY

The metabolism of exogenous tetramethylene 1,4- $^{14}\text{C}$ -labelled polyamines spermidine and spermine was studied in the rat

1 Spermidine is a precursor in the biosynthesis of spermine in rat tissues. The synthesis of spermine was most intensive in the organs where the molar ratio of spermidine to spermine was the lowest. The converse reaction, the degradation of exogenous spermine to spermidine, was also demonstrated. This conversion was marked in tissues with a high molar ratio. The formation of spermidine from exogenous spermine was more prominent at the age of 3 and 5 weeks in the liver, kidneys and pancreas than conversely, but there was practically no difference at the age of 8 and 30 weeks in this reversible reaction.

2 In the liver and kidneys the elimination of exogenous spermine was slower than that of spermidine. In the liver the spermine synthesized from exogenous spermidine was also eliminated more slowly than the spermidine formed from labelled spermine. 1 per cent of the spermidine and 4 per cent of the spermine radioactivity was degraded to respiratory  $\text{CO}_2$  during the first 12 hours after intraperitoneal injection. 7 per cent of the administered radioactivity derived from  $^{14}\text{C}$ -spermidine and 2 per cent of that from  $^{14}\text{C}$ -spermine was excreted into the urine under similar experimental conditions. The labelled compounds in the urine were not identified although they gave free putrescine and polyamines with the label during acid hydrolysis. No free labelled polyamines could be found in the unhydrolyzed urine.

3 A part of the perorally administered 1,4- $^{14}\text{C}$  spermidine and 1,4- $^{14}\text{C}$  spermine could be absorbed from the intestine. About 6 per cent of the spermidine was maximally found in the liver but only 0.4 per cent when  $^{14}\text{C}$  spermine was given *per os*.

4 In the regenerating liver the incorporation of radioactivity from 1,4- $^{14}\text{C}$ -spermidine to putrescine was observed to be active at 2 to 6 hours after partial hepatectomy. The formation of spermidine from 1,4- $^{14}\text{C}$  spermine was also increased immediately and markedly after operation. The highest values were obtained at 8 to 24 hours. The incorporation of 1,4- $^{14}\text{C}$



spermidine to spermine did not rise at 4 to 12 hours. In this reaction increased values were noticeable 24 and 48 hours after partial hepatectomy.

■ Daily administration of ethionine caused nearly complete inhibition on spermine synthesis from 1-4  $^{14}\text{C}$  spermidine. This treatment had no effect on the converse reaction from 1-4  $^{14}\text{C}$  spermine to spermidine.

6. Starvation of 1 to 4 days caused a decrease in the spermidine and a slight increase in the spermine concentration. Starvation increased the degradation of 1-4  $^{14}\text{C}$  spermine to spermidine but had no effect on the converse reaction.

## REFERENCES

- AMES B N and D T DUBIN The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid *J biol Chem* 1960 235 769-775
- BACHRACH U Formation of pyrroline and propane diamine from spermidine by extracts of *Serratia marcescens* *Nature (Lond)* 1962 a 194 377-378
- BACHRACH U Spermidine oxidase from *Serratia marcescens* *J biol Chem* 1962 b 237 3443-3448
- BACHRACH U and R BAIOR The formation of spermidine from spermine by serum amine oxidase *Biochim biophys Acta* 1960 40 545
- BACHRACH U S PERSKY and S RAZIN Metabolism of amines - The oxidation of natural polyamines by *Mycobacterium smegmatis* *Biochem J* 1960 76 306-310
- BACHRACH U and E ROBINSON Occurrence of spermine in sera of cancer bearing individuals *Israel J med Sci* 1965 1 247-250
- BARTELS H and H J HOHOPST Zur Wirkung des Aethionins auf den Metabolitusstatus der Rattenleber *Biochim biophys Acta* 1963 71 214-216
- BEER J Z O ROSIEK and J M SABLINSKI Niektóre poliaminy alifatyczne i ich znaczenie biologiczne *Po lepy Biochem* 1966 12 63-94
- BLASCHKO H The amine oxidase of mammalian blood plasma In *Advances in Comparative Physiology and Biochemistry* Vol 1 Ed O Lowenstein Acad Press London 1962 67-116
- BRAY G A A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter *Analyt Biochem* 1960 1 279-285
- BREWER E N and H P PUSCH Control of DNA replication Effect of spermine on DNA polymerase activity in nuclei isolated from *Physarum polycephalum* *Biochem biophys Res Commun* 1966 25 579-584
- BUCHER N L P Regeneration of mammalian liver In *International Review of Cytology* Vol 15 Ed G H Bourne and J F Danielli Acad Press New York 1963 245-300
- BUSCH S P CHAMBON P MANDEL and J D WEILL The effect of partial hepatectomy on the ribonucleic acid polymerase of rat liver *Biochem biophys Res Commun* 1962 7 255-258
- CALDARERA C M R BARBIROLI and G MORUZZI Polyamines and nucleic acids during development of the chick embryo *Biochem J* 1965 97 84-88
- CALDARERA C M R BUDINI B BARBIROLI and A RABBI The effect of ethionine on the free nucleotides in rat liver *Cancer Res* 1962 22 1026-1030
- CALDARERA C M C COZZANI and M S MORUZZI Polyamines and nucleic acids in rat liver subjected to ionizing radiation *Experientia* 1966 22 579-580
- CANTONI G L and J DURELL Activation of methionine for transmethylation II The methionine-activating enzyme Studie on the mechanism of the reaction *J biol Chem* 1957 225 1033-1048
- CHPSTENSEN H N J T ROTHWELL R A SEARS and J A STREICHER Association between rapid growth and elevated cell concentrations of amino acids II In regenerating liver after partial hepatectomy in the rat *J biol Chem* 1948 175 101-105
- COHEN S S and J LICHTENSTEIN Polyamines and ribosome structure *J biol Chem* 1960 235 1112-1116
- COHEN S S U HOFFNER M JANSEN M MOORE and A RAINA Poly

- amines RNA synthesis and streptomycin lethality in a relaxed mutant of *E coli* strain 25 TAU *Proc nat Acad Sci (Wash)* 1967 57 721-728
- COHEN S S and A RAINA Some interrelations of natural polyamines and nucleic acids in growing and virus infected bacteria *Rutgers Symposium* Sept 1966 *in press*
- COLBOURN J L B H WITHERSPOON and E J HERBST Effect of intracellular spermine on ribosomes in *Escherichia coli* *Biochim biophys Acta* 1961 49 422-424
- DUBIN D T Evidence for conjugates between polyamines and glutathione in *E coli* *Biochem biophys Res Commun* 1959 1 262-265
- DUBIN D T and B M ROSENTHAL The acetylation of polyamines in *Escherichia coli* *J biol Chem* 1960 a 235 776-782
- DUBIN D T and S M ROSENTHAL Polyamine glutathione conjugates in *E coli* *Fed Proc* 1960 b 19 1
- DUDLEY H W and O ROSENHEIM Notes on spermine *Biochem J* 1925 19 1034-1036
- DYKSTRA W G and E J HERBST Spermidine in regenerating liver relation to rapid synthesis of ribonucleic acid *Science* 1965 149 428-429
- FARBER E K H SHULL S VILLA TREVINO B LOMBARDI and M THOMAS Biochemical pathology of acute hepatic adenosinetriphosphate deficiency *Nature (Lond)* 1964 203 54-40
- FERRARI V and R D HARKNESS Free amino acids in liver and blood after partial hepatectomy in normal and adrenalectomized rats *J Physiol* 1954 124 443-465
- FINK K R E CLINE and R M FINK Paper chromatography of several classes of compounds correlated Rf values in a variety of solvent systems *Analyt Chem* 1963 35 380-398
- FOX C F W S ROBINSON R HASELKORN and S B WEISS Enzymic synthesis of ribonucleic acid III The ribonucleic acid primed synthesis of ribonucleic acid with *Micrococcus lysodeikticus* ribonucleic acid polymerase *J biol Chem* 1964 239 186-195
- FOX C F and S B WEISS Enzymic synthesis of ribonucleic acid II Properties of the deoxyribonucleic acid primed reaction with *Micrococcus lysodeikticus* ribonucleic acid polymerase *J biol Chem* 1964 239 175-185
- FRIEDMAN M E and U BACHRACH Inhibition of protein synthesis by spermine in growing cells of *Staphylococcus aureus* *J Bact* 1966 92 49-55
- FUJIOKA M M KOGA and I LIEBERMAN Metabolism of ribonucleic acid after partial hepatectomy *J biol Chem* 1963 238 3401-3406
- GOLDSTEIN J The effect of spermine on the accumulation of nucleic acids and protein in mammalian cells *Exp Cell Res* 1965 37 494-497
- GOLDSTEIN J Persistence of RNA to thermal denaturation in the presence of polyamines *Biochim biophys Acta* 1966 123 620-625
- GREENE R C Incorporation of the carbon chain of methionine into spermidine *J Am chem Soc* 1957 79 39-9
- HECHT L I and V R POTTER Nucleic acid metabolism in regenerating rat liver I The rate of deoxyribonucleic acid synthesis *in vivo* *Cancer Res* 1956 16 998-999
- HECHT L I and V R POTTER Nucleic acid metabolism in regenerating rat liver V Comparison of results *in vivo* and in tissue slices *Cancer Res* 1958 18 186-19
- HERBST E J and E E SNELL Putrescine and related compounds as growth factors for *Hemophilus parainfluenzae* 7901 *J biol Chem* 1949 181 47-54
- HERSHKO A S AMOZ and J MAGER Effect of polyamines and divalent metals on *in vitro* incorporation of amino acids into ribonucleoprotein particles *Biochem biophys Res Commun* 1961 5 46-51
- HIGGINS G M and R M ANDERSON Experimental pathology of the liver Restoration of the liver in the white rat following partial surgical removal *Arch Path* 1931 1- 186-202

- HIRSCH J G The essential participation of an enzyme in the inhibition of growth of tubercle bacilli by spermine *J exp Med* 1953a 97 327-343
- HIRSCH J G Spermine oxidase an amine oxidase with specificity for spermine and spermidine *J exp Med* 1953b 97 345-355
- JACKSON E L and S M ROSENTHAL The preparation of C<sup>14</sup> labelled spermine and C<sup>14</sup> labelled spermidine *J org Chem* 1960 25 1055-1056
- JÄNNE J Effect of growth hormone on putrescine and polyamine synthesis in rat liver *Scand J clin Lab Invest* in press
- JÄNNE J and A RAINA Further observations on the biosynthesis of polyamines in regenerating rat liver *Acta chem scand* 1966 20 1174-1176
- JÄNNE J, A RAINA and M SIIMES Spermidine and spermine in rat tissues at different ages *Acta physiol scand* 1964 62 352-358
- KRAKOW J S Ribonucleic acid polymerase of *Aerobacter tinelandii* III Effect of polyamines *Biochim biophys Acta* 1963 72 466-571
- KREMNZER L T and R M STARR Effect of ethionine on histamine and spermidine tissue levels *Fed Proc* 1966 25 559
- LEWENHOECK A Observaciones D Anthoni Lewenhoeck de Natis = semini genitali *Anomalculia Phil Trans* 1678 12 1040-1043
- LIEBERMAN I P KANE and J SHOTT The portal vein and the control of liver ribonucleic acid metabolism *J biol Chem* 1965 240 3140-3144
- MANDEL M The interaction of spermine and native deoxyribonucleic acid *J molec Biol* 1962 5 435-441
- MARTIN R G and B N AMES The effect of polyamines and poly U size on phenylalanine incorporation *Proc nat Acad Sci (Wash)* 1962 48 2171-2178
- MENYHART J and L SIMON Acute circulatory consequences of partial hepatectomy *Acta physiol Acad Sci hung* 1966 30 161-168
- MICHAELSON I A Purification of brain histamine for fluorometric assay *Fed Proc* 1966 25 691
- MILLS J and D T DUBIN Some effect of spermine on *Escherichia coli* *Mol Pharmacol* 1966 2 311-318
- MITRA S and P KAESBERG Interaction of polyamines with turnip yellow mosaic virus RNA *Biochem biophys Res Commun* 1965 21 146-151
- MODY U S BULBA O HOLOWECKY and J A STEKOL The availability of the adenine moiety of S adenosyl derivatives of methionine and ethionine for the synthesis of liver AMP ADP ATP and RNA in male and female rats *Fed Proc* 1963 22 471
- MORRIS D R and A B PAPDEE A biosynthetic ornithine decarboxylase in *Escherichia coli* *Biochem biophys Res Commun* 1965 20 697-702
- MORRIS D R and A B PAPDEE Multiple pathways of putrescine biosynthesis in *Escherichia coli* *J biol Chem* 1966 241 3129-3135
- NEISCH W J P Liver glutathione and polyamines in hepatocarcinogen treated rats *Biochem Pharmacol* 1967 16 163-173
- NYGAARD O and H P RUSCH Incorporation of radioactive phosphate into nucleic acids of regenerating rat liver *Cancer Res* 1955 15 240-245
- O'BRIEN R L J G OLENICK and F E HAHN Reactions of quinone chloroquine and quinacrine with DNA and their effects on the DNA and RNA polymerase reactions *Proc nat Acad Sci (Wash)* 1966 55 1511-1517
- OCHOA M Jr and I B WEINSTEIN Polypeptide synthesis in a subcellular system derived from the L 1210 mouse ascites leukemia *J biol Chem* 1964 239 3834-384
- OCHOA M Jr and I B WEINSTEIN Spermine inhibition of polypeptide synthesis in a subcellular system derived from the L 1210 mouse ascites leukemia *Biochim Biophys Acta* 1965 95 176-179
- OLIVER I T F J BALLARD J SHIELD and P J BENTLEY Liver growth in the early postpartum rat *Develop Biol* 1962 4 108-116
- PADMANABHAN M and K. NIM Oxidation of spermidine by a *Pseudomonas* *Biochem biophys Res Commun* 1965 19 1-5

- PERRY T L & HANSEN J G FOULKS and G M LING Aliphatic and aromatic amines of cat brain *J Neurochem* 1965 12 397-405
- RAINA A Spermin- and spermidine in normal human blood *Scand J clin Lab Invest* 1962a 14 318-319
- RAINA A Biosynthesis of spermine and spermidine in the developing chick embryo *Acta chem scand* 1962a 16 2463-2464
- RAINA A Studies on the determination of spermidine and spermine and their metabolism in the developing chick embryo *Acta physiol scand* 1963 60 suppl 218
- RAINA A On the biosynthesis of spermidine and spermine in the rat *Acta chem scand* 1964 18 2188-2189
- RAINA A and S S COHEN Polyamines and RNA synthesis in a polyauxotrophic strain of *E coli* *Proc nat Acad Sci (Wash)* 1966 55 1587-1593
- RAINA A J JÄNNE and M SIIMES Effect of ethionine on spermidine spermine and adenine nucleotides in rat liver *Acta chem scand* 1964 18 1804-1806
- RAINA A J JÄNNE and M SIIMES Polyamines and nucleic acids in regenerating rat liver In *Abstracts of the 2nd Meeting at Federation of European Biochemical Societies* Vienna 1965
- RAINA A J JÄNNE and M SIIMES Stimulation of polyamine synthesis in relation to nucleic acids in regenerating rat liver *Biochim biophys Acta* 1966 123 197-201
- RAINA A and T TELARANTA Association of polyamines and RNA in isolated subcellular particles from rat liver *Biochim biophys Acta* 1967 138 200-203
- RAZIN S U BACHRACH and I GERY Formation of  $\beta$  alanine from spermine and spermidine by *Pseudomonas aeruginosa* *Nature* 1958 182 700-701
- RAZIN S I GERY and U BACHRACH The degradation of natural polyamines and diamines by bacteria *Biochem J* 1959 72 551-558
- RAZIN S and R ROZANSKY Mechanism of the antibacterial action of spermine *Arch Biochem* 1959 81 36-54
- RO T S and H BUSCH Studies on RNA polymerase of nuclear fractions of regenerating liver *Biochim biophys Acta* 1967 134 184-187
- ROSENTHAL S M and D T DUBIN Metabolism of polyamines by *Staphylococcus* *J Bact* 1962 83 859-863
- ROSENTHAL S M and C W TABOR The pharmacology of spermine and spermidine Distribution and excretion *J Pharmacol exp Ther* 1956 116 132-138
- POULET F and E A ZELLE Über die Diamin oxydase der Smegmabazillen *Helv chim acta* 1945 8 130-1342
- PAIHA N J JÄNNE and J SUIHKONEN Effect of partial hepatectomy on enzymes concerned with urea synthesis in rat liver *Scand J clin Lab Invest* in press
- SCHLENK F In *Transmethylation and Methionine Biosynthesis* Ed S K Shapiro and F Schlenk The University of Chicago Press Chicago 1965 p 53
- SCHNEIDER J H and V R POTTER Alternative pathways of glucose metabolism III The incorporation of radioactivity from glucose C14 into the nucleic acids of regenerating rat liver *Cancer Res* 1957 17 701-706
- SHIMIZU H Y KAKIMOTO and I SANO The determination and distribution of polyamines in mammalian nervous system *J Pharmacol exp Ther* 1964 143 199-204
- SHIMIZU H Y KAKIMOTO and I SANO Changes in concentrations of polyamines in the developing mouse brain *Nature (Lond)* 1965a 207 1196-1197
- SHIMIZU H Y KAKIMOTO and I SANO Spermine and spermidine in blood cells and a possible occurrence of spermine in cell nucleus *Arch Biochem* 1965b 110 368-372
- SHULL K H Hepatic phosphorylase and adenosine triphosphate levels in ethionine treated rats *J biol Chem* 196 237 1734-1735

- SHULL K H J McCONOMY M VOGT A CASTILLO and E FARBER On the mechanism of induction of hepatic adenosine triphosphate deficiency by ethionine *J Biol Chem* 1966 241 5060-5070
- SIIMES M Incorporation of radioactivity from spermidine C<sub>14</sub> into spermine in rat liver In *Abstracts of the 3rd Meeting at Federation of European Biochemical Societies* Warsaw 1966
- SIIMES M The interconversion of polyamines in growing rat tissues *Scand J Clin Lab Invest* in press
- SIIMES M and J JÄNNE Polyamines and their biosynthesis in Ehrlich ascites cells *Acta chem scand* in press
- SILVERMAN M and E A EVANS Jr The effect of spermine spermidine and other polyamines on the growth inhibition of *Escherichia coli* by atabrine *J Biol Chem* 1944 154 5-1-34
- SIMON E J S S COHEN and A RAINA Polyamines and inhibition of RNA synthesis *Biochem biophys Res Commun* 1966 24 482-488
- STEKOL J A Biochemical basis for ethionine effects on tissue In *Advances in Enzymology* Vol 25 Ed F F Nord Interscience Publishers New York 1963 369-392
- STEKOL J A U MODY E BEDRAK S KELLER and J PERRY Oxidative phosphorylation with liver mitochondria in ethionine fed rats *Fed Proc* 1960 19 37
- TABOR C W Adenosylmethionine decarboxylase In *Methods in Enzymology* Vol 5 Ed S P Colowick and N O Kaplan Acad Press New York 1962 a 756-760
- TABOR C W Propylamine transferase (Spermidine synthesis) In *Methods in Enzymology* Vol 5 Ed S P Colowick and N O Kaplan Acad Press New York 1962 b 761-765
- TABOR C W and S M ROSENTHAL Pharmacology of spermine and spermidine Some effects on animals and bacteria *J Pharmacol exp Ther* 1956 116 139-155
- TABOR C W and H TABOR Transport systems for 1,4-diaminobutane spermidine and spermine in *Escherichia coli* *J Biol Chem* 1966 241 3714-3723
- TABOR C W H TABOR and U BACHRACH Identification of the amino aldehydes produced by the oxidation of spermine and spermidine with purified plasma amine oxidase *J Biol Chem* 1964 239 2194-2203
- TABOR C W H TABOR and L de MEIS Studies on the structure and biosynthesis of a spermidine glutathione derivative of *E. coli* *Fed Proc* 1966 25 709
- TABOR C W H TABOR and S M ROSENTHAL Purification of amine oxidase from beef plasma *J Biol Chem* 1954 208 645-661
- TABOR H The protective effect of spermine and other polyamines against heat denaturation of deoxyribonucleic acid *Biochemistry* 1962 1 496-501
- TABOR H S M ROSENTHAL and C W TABOR Biosynthesis of spermidine and spermine from putrescine *Fed Proc* 1956 15 367
- TABOR H S M ROSENTHAL and C W TABOR The role of putrescine and methionine in the enzymatic synthesis of spermidine in *Escherichia coli* extracts *J Am Chem Soc* 1957 79 2978-2979
- TABOR H S M ROSENTHAL and C W TABOR The biosynthesis of spermidine and spermine from putrescine and methionine *J Biol Chem* 1958 233 907-914
- TABOR H and C W TABOR Further purification of the enzyme concerned with the biosynthesis of spermidine in *E. coli* *Fed Proc* 1960 19 6
- TABOR H and C W TABOR Metabolically-dependent uptake of C<sub>14</sub> spermidine by *E. coli* *Fed Proc* 1964 a 3 516
- TABOR H and C W TABOR Spermidine spermine and related amines *Pharmacol Rev* 1964 b 16 45-300









ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 299

Carl Koller and the Development of  
Local Anesthesia

BY  
G LILJESTRAND

STOCKHOLM 1967



ACTA PHYSIOLOGICA SCANDINAVICA

*Supplementum 299*

From the Department of Pharmacology The Caroline Institute  
Stockholm Sweden

Carl Koller and the Development of  
Local Anesthesia

BY

G LILJESTRAND

*En ritu Professor of Pharmacology*

STOCKHOLM 1967

Stockholm 1967  
Kungl. Boktryckeriet P. A. Norstedt & Söner

In 1963 in collaboration with Dr. now Professor H. Holmstedt, the author published a book entitled *Readings in Pharmacology* (28). It was an attempt to give a brief survey of the development of pharmacology from ancient times to the present day by direct quotations from more or less fundamental publications (translated into English when necessary). They were preceded by brief biographical notes on the scientists concerned and joined together so as to cover the more important fields. One of the twelve chapters dealt with local anesthesia and it contained among other things a considerable part of the lecture given on that subject especially with regard to the eye by the young Austrian physician Carl Koller (1857—1944) on October 17 1884 before the Wiener medizinische Gesellschaft (Vienna Medical Society). This work aroused in the author a lively interest in Koller his work and his life. I accidentally met one of my earlier fellow professors at the Caroline Institute Dr. J. Wilhelm Nordenson M.D. D.Sc. (1883—1965) at the time Emeritus Professor of Ophthalmology. I knew that he had met Koller in New York (59) and he told me a great deal about that meeting.

My colleague had obtained a considerable part of his scientific training with Allvar Gullstrand (1862—1930) the first professor of Ophthalmology at the University of Uppsala (54) well-known for his many important contributions to our knowledge of the functions of the eye as well as numerous practical applications. In 1911 he was awarded the Nobel Prize in Physiology & Medicine for his work on the dioptrics of the eye. Three years later he was appointed to a personal professorship in Physical and Physiological Optics at the University of Uppsala which he held until he became Emeritus in 1927. The ordinary chair of Ophthalmology became occupied in 1915 by C. F. Lindahl M.D. (1874—1943) a chronic disease however compelled him to take temporary leave in the course of 1922 and Nordenson was charged to act as his substitute. The health of Professor Lindahl deteriorated however and in 1927 he retired completely and at the end of that year Nordenson became his successor. Soon afterwards (in 1931) he accepted a call to the corresponding chair at the Caroline Institute in Stockholm. In 1948 he retired having reached the age limit.



Carl Koller in 1885  
(After E. Tesky)

Contact with American medicine had been practically impossible during the First World War and it took several years before it became normal again. Professor Gullstrand was interested in making his own work known on the other side of the Atlantic and in studying the progress made there. He also planned to try to pave the way for the renewal of international cooperation in his science; consequently he attended as an official delegate of Sweden the

International Congress of Ophthalmology (23) which took place in Washington D.C. on April 25—28, 1922. He was accompanied by his former assistant Nordenson. On April 25 at the Congress Gullstrand gave a much appreciated communication entitled "On diaphragm lamps in Ophthalmology" where he described the slit lamp and the aspheric lens as well as the hole or diaphragmatic lamp. He also discussed focal illumination and several kinds of reflex free ophthalmoscopy, from the large instrument with stereoscopic vision to the simplified one as well as the centric photography of the fundus oculi (27: 69—80). Next day Professor Gullstrand gave a demonstration of

Diaphragm Illumination and Dr. Nordenson another about Centric Photography of the Fundus Oculi (27: 69). According to Nordenson's account it might have been in this connection that he first met Koller. With his customary civility Nordenson was introducing himself to those colleagues attending the demonstrations when he suddenly heard the name of Koller. A collection



The middle aged Koller

of letters from Koller to Erik Nordenson (1847—1919) M.D. a well known ophthalmologist and the father of Wilhelm who inherited those letters had made him well acquainted with Koller's work so that contact rapidly became established. Koller's name appears on the membership list of the Congress (23 19) but without the star indicating his presence so it is possible that he only attended the demonstrations given elsewhere and he may even have gone in order to meet Nordenson. Since Nordenson in his biography of Gullstrand (54 329) tells us that the demonstrations by Gullstrand and Nordenson were also given in New York before a selected group at a session arranged by the local committee somewhat earlier than the Congress itself it might have been in that connection that he saw Koller. It is certain however that a meeting between the two took place in New York. This was mentioned in a speech by Nordenson which he delivered as Dean of the medical faculty at Uppsala on May 30 1931 (53) when conferring the degree of M.D. In commemoration of the first similar conferment in Sweden 250 years earlier, he then gave a lecture about the work of Koller on cocaine and mentioned his visit to him in New York. This is confirmed by a letter from Koller to his daughter Mrs H. Koller Becker quoted in her publication mentioned below (38 366) where he tells her that Nordenson (as chairman of the International Council of Ophthalmology 1933—1950) had arranged for Koller's preliminary



communication on cocaine to be published in an English translation in 1934 on the 50th anniversary of his great discovery and also that special attention was given to the jubilee at the meeting of German ophthalmologists in 1934. Besides the letter contains the following lines about Nordenson who mother says, is the best looking man she ever met which indicated that Nordenson had been at Koller's home.

I got the opportunity to read the letters from Koller to Erik Nordenson found them very interesting and proposed that they should be published. Wilhelm Nordenson gladly consented but since he felt very tired he asked me to attend to this matter. At about this time Dr Holmstedt and I received a letter, dated August 21, 1964 from a lady unknown to us who lived in Highland Park Ill. She was the above mentioned daughter of Carl Koller. She told us that P. Knoepfel Professor of Pharmacology at the University of Louisville, had called her attention to our book and what she was kind enough to characterize as its excellent and accurate account of the discovery of local anesthesia in surgery by my father. Enclosed were two copies of a comprehensive article "Carl Koller and Cocaine" that she had written and published at about the same time that our book appeared (38). Her report which will often be quoted in the following pages turned out to be a very well written and carefully documented account. To a great extent it is founded on papers left behind by her father among them several earlier unpublished letters from Sigmund Freud to him as well as letters from Koller to his daughter. The first named letters had been translated into English with the cooperation of Dr Ernst Freud a son of the great psychoanalyst. When reading the article one could hardly avoid finding that the case of Koller was to a certain extent analogous to several others which have bestowed on the Vienna medical faculty an unenviable notoriety e.g. those of Semmelweis and of Landsteiner. In this situation it seemed desirable to get into print as soon as possible the letters to Erik Nordenson who seems to have been a kindred spirit to Koller with regard to independent judgment as well as fearlessness. At the end of November 1965 the relevant number of the journal appeared (46) — the cover has the date of September but that was evidently what had been hoped for which as is well known does not always coincide with the result. In the following we will find that particulars about the date of publication ought sometimes be taken with some caution.

From Koller's letters one gets the impression of a highly intelligent and temperamental person with strong scientific interests and independent judgment. But it also becomes evident that Koller at the beginning of this time (1885—1886) felt himself persecuted in Vienna and depressed. The causes of this will be illustrated later in this paper. He probably met Dr Erik Norden

son at the *Versammlung deutscher Naturforscher und Ärzte* (Assembly of German natural scientists and physicians) in Strassburg in September 1885. In a letter (38, 354) Freud had advised him to go there—partly because there may be a market in which someone would buy you. Erik Nordenson who had been abroad for many years studying ophthalmology during the period 1883—1887 lived in Göttingen engaged in an extensive investigation of the detachment of the retina ( *ablatio retinae* ) at the clinic of Professor Th. Leber. Nordenson had attended the meeting at Heidelberg in September 1884 when Koller's discovery of the value of cocaine for anesthesia of the eye was reported and demonstrated. It was no doubt Nordenson who arranged a sojourn for Koller at Leber and it was also certainly his personal acquaintance with Donders and Snellen in Utrecht which led to a more than a year long engagement there. The zest for life now returned to Koller and Freud wrote (38, 357)

"With the greatest pleasure I see from your letter what a warm interest you take in me and I conclude further that a gratifying change has taken place in you since I saw you last at the peak of your illness which now that I am riper in experience

— Freud had just spent several highly appreciated months in Paris with Charcot —

I can certainly diagnose as *neurasthenia*

Both Freud and Nordenson advised Koller to emigrate to the USA and after some hesitation which was removed after a visit to Vienna, he departed via London where he spent some days for New York arriving there May 18, 1889. He spent the following 55 years in that city as a highly appreciated eye practitioner and consultant at several hospitals. His old inclination to scientific work remained and in spite of the somewhat unfavourable circumstances he published a number of papers demonstrating his keen power of observation, his energy and broad knowledge. Thus he described several rare and even fatal eye diseases, he demonstrated the nerves of the cornea and he also took up problems about astigmatism, its determination and the form of the retinal images in this situation. He contributed to the therapy of certain eye diseases e.g. by blood letting. Furthermore he constructed an improved system of illumination for the electric ophthalmoscope (1918). A paper on cocaine in eye operations (35) will be considered later on.

Like many others perhaps more in those days than now, during the beginning of his stay in New York, Koller felt convinced that the Americans were greatly inferior to the Europeans with regard to education and civilization but he soon learnt to appreciate the American people, their great

hospitality and readiness to help love of freedom and efficiency without overlooking the shortcomings which must necessarily be incident to such a heterogeneous nation

It now seemed that a thorough analysis of the role of Koller would be desirable from the standpoint of the pharmacologist and consequently when I was asked to write an introductory chapter about the history of local anesthesia for the volume on local anesthesia for the great International Encyclopedia of Pharmacology and Therapeutics I readily accepted this invitation (47) It is to some extent on the analysis made there that this report rests though many new details are given here

The introduction of general anesthesia or narcosis is rightly considered as one of the greatest advances made within medicine in the 19th century together with antiseptics and asepsis it forms the foundation for the splendid development of surgery in our time Here an exact dating can be given viz Thomas Green Morton's famous demonstration of the practical usefulness of ether narcosis for operative purposes taking place at the Massachusetts General Hospital at Boston on October 16 1846 The following year James Simpson Professor of Obstetrics at Edinburgh proposed the use of chloroform instead of ether and later on there have been continuous endeavours to find new means for general narcosis with lessened risks To day this speciality has advanced a great deal in many cases certain derivatives of barbituric acid are in the foreground owing to the quick and easy induction—they are given intravenously—and the short duration of their action It is characteristic for the situation however that in 1848 thus shortly after the introduction of chloroform Simpson himself pointed out (67) that if we were able to call forth local anesthesia without the unconsciousness that accompanies general narcosis many would greet this as still greater progress He also made some crude experiments to reach the desirable goal using the vapours of chloroform ether carbon disulphide and even hydrocyanic acid Some years later the surgeon A. Richet in Paris (61), father of the Nobel laureate Ch. Richet emphasized the danger of chloroform narcosis even in the hands of experienced physicians and tried with some success to use ether for local anesthesia but this time employing its cooling effect when vaporizing it

Just as general anesthetics have old ancestry in the form of opium Mandragora and palm wine all of them dangerous at a time when great uncertainty prevailed regarding the dosage attempts have been made for centuries to call forth local anesthesia sometimes not without a certain amount of success I will only give a few hints here

It is well known that hypnosis and several of its forerunners such as incantations amulets the influence of sorcerers and medicine men might give a

blunting of pain sensation and at least during smaller operations be of considerable value. For a long time it was also believed that continuous pressure on certain nerve trunks ought to be used as a means of producing anesthesia in the innervated area usually an extremity. As an example the short description given by Ambroise Pare (1510—1590) the famous military surgeon of the 16th century may be mentioned. He emphasized that powerful squeezing gives rise to three advantages viz better possibilities of covering the bone stump after an amputation, the stopping of bleeding and a reduction of pain (57). It is characteristic of Pare's good judgment that he mentioned the reduction of pain but not its disappearance. The explanation of the unsatisfactory results of the squeezing has been given in modern times by Gasser and Erlanger who received the Nobel Prize in Physiology or Medicine (1944) for their discoveries relating to the highly differentiated functions of single nerve fibres. Using the cathode ray oscillograph which they had introduced into physiology they proved that the conduction velocity in the single fibres is approximately a linear function of the diameter of the fibres concerned. Blocking the conduction with pressure was found to give effect at first on fibres with large diameter i.e. motor fibres whereas the fine fibres conducting pain impulses were affected last. On the other hand these fine fibres were affected first by a cocaine solution presumably due to the relatively great surface area as well as by cooling (22). As already indicated cooling has been used in order to obtain local anesthesia. Here another great military surgeon D. J. Larrey (1766—1842) Napoleon's head surgeon in La grande armée has published practical results of considerable importance (42). Thus he described that he had operated mainly performing amputations under very cold conditions ( $-19^{\circ}\text{C}$ ) when his assistants were hardly able to hold the instruments but dropped them to the ground. He found it striking that the patients recovered with unexpected rapidity and immediately after the operations felt much relieved. Larrey's description seems to be the first demonstration of the valuable effect of hypothermia during operations probably because shock is avoided and the central nervous system is relatively little affected.

As we all know it is with the aid of chemicals that local anesthesia has gained its greatest victories. The progress is naturally tied to the development of chemistry. Alcohol was the first of the substances used for this purpose sometimes it is used even to-day e.g. for injection in the nerve trunk in unbearable neuralgia of the trigeminal nerve. But in this case high concentrations are used (over 50%) and the nerve is thus killed. The starting point for modern local anesthesia is as is well known the alkaloid cocaine for the first time obtained in the pure state from the leaves of the Coca bush by A. Niemann (52) in the laboratory of F. Wohler in Göttingen. Niemann also

morphine. The consequence was the one so often met with—he became a morphine addict. His friends tried to help him in different ways and when Freud had found reports in American literature about the successful use of cocaine in such cases in order to get rid of the addiction, he decided to try such treatment on his friend. The result at first seemed excellent and Freud felt convinced that here was a great discovery that he would like to exploit and develop. As time went on, however, the patient showed an increased need for the new remedy and soon chronic cocaineism had succeeded the morphinism. This seems to have been the case already before 1886 for Koller tells us in a lecture from 1940 (38/326) that ‘many a night have I spent with him watching him dig imaginary insects out of his skin in his sensory hallucinations’, thus typical symptoms of chronic cocaineism had developed. Freud who as mentioned collaborated with Koller though nothing of value resulted was now deeply interested in the wonderful drug cocaine and went through the literature the result being published in a summing up which from several points of view is excellent and came in print in July 1884 (19). Recently an English translation has become available (19b). He there refers in detail to the animal experiments already performed by Moreno and by von Anrep from which it appeared that at first a stimulating and later a paralysing effect was obtained. Of course he also gave much attention to the general effects on man as described e.g. in the classical papers by Mantegazza and others as well as the findings of the old Christison in experiments on himself where a considerable improvement of the capacity for work became established with cocaine e.g. during mountaineering—in good agreement with the experiences with coca leaves of the natives in South America. Freud mentioned after Niemann that cocaine produces an anesthetic effect on the mucous membranes (19b/5). With regard to his own observations on this point he wrote (19b/9)

A few minutes after taking cocaine one experiences a sudden exhilaration and feeling of lightness. One feels a certain fullness on the lips and palate followed by a feeling of warmth on the lips and cold in the throat. On other occasions the predominant feeling is a rather pleasant coolness in the mouth and throat.

The effects with regard to the disappearance of pain sensations after pin pricks is not mentioned. It is also characteristic that Freud does not refer to or comment upon the observations on themselves by Moreno or von Anrep quoted above nor of their conclusions about the possibility that cocaine might be used to induce local anaesthesia e.g. by direct injection.

In the last chapter of his review Freud summarized the different forms in which cocaine might be used with advantage in therapeutics. The following possibilities are mentioned: a) as a stimulant b) for digestive disorders of the stomach c) in cachexia d) in the treatment of morphine and alcohol addic-



**The measurement of secretion and absorption by intestinal loops in conscious dogs** By L M VAUGHAN WILLIAMS *Department of Pharmacology, University of Oxford*

An operation for the introduction of cannulae into both ends of a Thiry Vella intestinal loop, and an apparatus for measuring the rate at which fluid is transported along it has already been described (Streeten & Vaughan Williams, 1951). The cannulae used were made of silver and it was not found possible to prevent the ultimate development of leaks around them. Recently, with

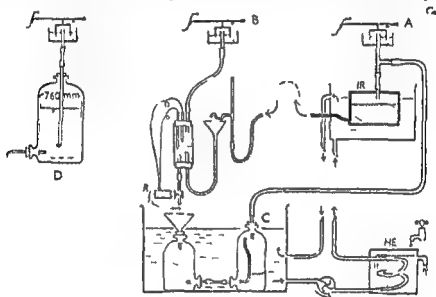


Fig 1

plastic cannulae of slightly modified design, such leakage has been eliminated. In the absence of any loss of fluid between the loop and recording equipment, it became possible to extend the method to the measurement of rates of secretion or absorption by the loop.

In principle the apparatus (Fig 1) is very simple. One float recorder (A) measures the rate at which fluid enters the loop and another (B) the rate at which it leaves.

Fluid flows from a reservoir whose height is adjustable, through a heat exchanger (not shown), which raises its temperature to  $38^{\circ}\text{C}$ , into the proximal end of the loop. It emerges from the distal end, and, if a sufficient force runs out of a side arm in a vertical glass tube, which is also adjustable. The volume of fluid expelled is recorded by a float recorder (B) connected to a glass cylinder which, when filled, is emptied by a relay (H).

The addition of another recorder to the apparatus allows the measurement of the rate of

tion e) as an aphrodisiac f) local application This last section, having played a special role in the discussion will be quoted in full

Cocaine and its salts have a marked anæsthetic effect when brought in contact with the skin and mucous membranes in concentrated solution this property suggests its occasional use as a local anæsthetic, especially in connection with affections of the mucous membranes According to Collin Ch Fauvel strongly recommended cocaine for treating diseases of the pharynx describing it as *le tenseur par excellence des cordes locales* Indeed the anæsthetic properties of cocaine should make it suitable for a good many further applications

Freud's description of the local actions of cocaine is both incorrect and incomplete thus demonstrating that his real interest in this aspect must have been rather insignificant in comparison to the general effects to which he obviously attached great weight Among the errors one first observes Freud's statement that cocaine has an anæsthetic action on the skin which he does not substantiate in any way from the literature There is in fact no such effect It is of special interest in this connection that Wagner (later known as Wagner Jauregg) in the beginning of 1886 published a paper (68) describing a method of obtaining anæsthesia of the intact skin with cocaine His starting point was a publication from the preceding year by Rusconi (62) who had written that painting the skin with a two per cent aqueous solution of cocaine hydrochloride within two minutes provoked an anæsthesia so that needle pricks were hardly felt and a piece of ice placed on the skin was only felt after a minute by the subject The effect became still stronger with a three per cent solution in alcohol With this painted on the skin deep seated pain as at parametritis could be abolished and it even permitted incisions and small operations without any pain One wonders whether Wagner took up the problem because he had observed the error made by Freud and preferred to discuss the question without mentioning the mistake of his friend Wagner stated that he had not succeeded in obtaining anæsthesia of the intact skin with cocaine solutions even after ten minutes and also using much stronger solutions By the aid of cataphoresis of cocaine solutions in water—the method had been introduced by Gartner—it became possible for Wagner to obtain complete anæsthesia of the skin so that an incision through it was not felt This communication by Wagner seems to have stimulated others and soon afterwards A Adamkiewicz (1) tried the method with chloroform and came to the conclusion that it was possible by cataphoresis to obtain skin anæsthesia also with this substance H Paschke and Wagner found this very improbable (58) since chloroform carries practically no electric current and they proved convincingly that the observations of Adamkiewicz had been erroneously interpreted (58) They warned directly against the local use of chloroform since it may give rise to



rather profound etching 'as we to our discomfort have convinced ourselves. But the anesthetic effect of cocaine is not the only mistake made by Freud the same is true with regard to his statement that cocaine and its salts have an anesthetic effect on mucous membranes since the effect in this case is practically restricted to the salts as discussed later (p 20). Furthermore Freud stated that to be efficient the solutions should be concentrated but as mentioned above von Anrep found good effect from 0.6—1 per cent solutions. The results from subcutaneous injections by Moreno and by von Anrep seem to have been completely unknown to Freud though he quoted both papers. In other connections Freud mentioned the subcutaneous applications of the substance for therapeutic purposes (19 b 15) but his curiosity seems to have been limited to the reduction of pain on other mucous membranes than those already tried in this direction. As a basis for the assertion that Freud has given Koller the impulse to his decisive experiments on the surgical application of cocaine anesthesia one must conclude that it is very meagre much more so than shown by the earlier literature.

Having finished his review Freud made a journey but before leaving Vienna he spoke to his friend Dr L. Konigstein assistant professor in ophthalmology asking him to take up the question of the practical use of cocaine in connection with diseases of the eye. Koller who had had practical personal experience of the numbing effect of cocaine hydrochloride on the tongue and the mucous membrane of the mouth during his experiments with Freud asked himself whether this might be the substance he had been looking for without success. He went to Stricker's laboratory and tried it first on a frog then a guinea pig a rabbit and a dog and when all these experiments clearly established its efficiency in producing local anesthesia corresponding trials were performed with the same success on himself and the assistant Dr Gartner (21 quoted from 38 331) who warmly congratulated him to the important finding.

Being naturally anxious to have his results known in scientific circles and the priority protected Koller wrote a brief summary and asked his friend Dr A. Breittauer from Trieste to read it at the meeting of the German ophthalmologists in Heidelberg on September 15 and 16 1844—economic difficulties made it impossible for Koller to go himself.

In this classical report now available also in English translation (33) Koller carefully described the anesthesia of the conjunctiva and the cornea after instillation of a few drops of a two per cent aqueous solution of cocaine hydrochloride into the conjunctival sac its rapid onset with the abolition of all reflexes from the cornea as well as its relatively slow disappearance. In animals he proved that the anesthetized cornea could be scratched or scraped even perforated or cauterized with silver nitrate until it became completely white.

and also be cut without any reaction from the animal. Also after the production of a foreign body keratitis the effect of cocaine was apparent. In man the local anesthesia from cocaine was accompanied by a widening of the palpebral fissure as well as for the pupil though the dilation of the pupil was not maximal. A slight paresis of the accommodation was also observed.

In conclusion Koller wrote

Perhaps it would not be presumptuous if I expressed the hope that cocaine can be used successfully as an anesthetic agent in removal of foreign bodies from the cornea in more extensive operations or as a narcotic in diseases of the cornea or conjunctiva. Since the experiments I have just described were carried out only during the past two weeks I must reserve for later publication a consideration of other papers which possibly concern this question and an evaluation of their contents. Vienna beginning of September 1884

This report a model of exact and concise description careful conclusions and hopeful optimism was very well received at the Heidelberg meeting especially after Dr. Brettauer had demonstrated the remarkable effect of a solution of cocaine hydrochloride on the eye of one of the patients from the Eye Clinic. The only person who expressed himself in the discussion was Dr. H. Schmidt Rimpler professor of ophthalmology at the University of Halle who said "The new substance will if it is borne out further be of the utmost importance for our operations." Dr. H. D. Noyes professor of ophthalmology in New York who had been present at the Heidelberg meeting immediately wrote a letter to an American colleague who tried cocaine on October 11 though the result was not made known before much later (50). But Noyes also published a report from the meeting in a well known journal (55) where he wrote

Perhaps the most notable thing was the exhibition to the Congress on one of the patients of the Heidelberg Eye Clinic of the extraordinary anesthetic power which a two per cent solution of muriate of cocaine had upon the cornea and conjunctiva when dropped into the eye. The momentous value of the discovery seems likely to prove to be in the eye practice of more significance than has been the discovery of anesthesia by chloroform or ether in general surgery or medicine.

The later publication indicated by Koller in the words quoted above took the form of a lecture read before the Wiener Medizinische Gesellschaft (Viennese Medical Society) on October 17 1884 and published soon afterwards (34). He could then state that his results had already been confirmed in various places in Germany. He now referred to some older observations of the numbing effect of cocaine hydrochloride on the tongue and the palate performed on himself by C. D. Schiörrf in 1862 (64). He further mentioned von Anrep's comprehensive account of experiments with cocaine and also

in contrast to Freud—that he had pointed out at the end of his paper that the anesthetic action of cocaine might be of importance

For us Viennese he continued - cocaine has become a favorite topic of discussion because of the thorough review and interesting work of my colleague at the Allgemeines Krankenhaus (General Hospital) Dr Sigmund Freud

Koller was also able to make some important additions to his first report such as ischemia of the conjunctiva after cocaine as well as widening of the palpebral fissure, occurring before the action on the muscles in the iris and the ciliary body and therefore attributed by Koller to removal of stimuli. He also concluded from the late effects on the pupil and the accommodation that a slow absorption of the cocaine solution had taken place. Still more important was the fact that Koller could refer to clinical observations—thanks to the kindness of Professor A. L. von Reuss who was the temporary chief of the second Eye Clinic at the hospital—which clearly demonstrated the practical value of cocaine solutions in the treatment of phlyctenular conjunctivitis with eruptions and ulcers of the cornea, as well as the prevention for a few hours of pain from cauterization of the lids with silver nitrate. He also had used cocaine successfully in several operations such as removal of foreign bodies from the cornea further in iridectomy and even an extraction of cataract without the patient feeling any pain.

Koller's lecture was followed at the same meeting by a communication from Königstein (see below). Professor Lesky in her valuable book (44 489) *Die Wiener medizinische Schule im 19. Jahrhundert* says that Königstein's contribution was delivered on October 19 thus 2 days after Koller's but there is no doubt that it occurred at the same date as Koller's lecture as seen from the minutes of the meeting (40) and also from Koller's own words (38 341). October 19 is the date of the publication of the first half of Königstein's lecture (39). At the same meeting (Oct. 17) Dr Hock and Professor Reuss confirmed the absolutely striking success with cocaine. The latter advised all ophthalmologists to make no operations before the application of cocaine. This must be applied directly on the cornea and for long time. It can be used also in glaucoma.

A week later (the minutes 40 131b have been wrongly dated to Oct. 25 instead of Oct. 24 cf Jelinek 30) Jelinek spoke in the society about painless operations in the laryngeal region under the influence of cocaine which he had tried at the advice of Koller. In the discussion Professor L. von Schrötter pointed out the value of the new method for laryngoscopy. A little later E. Frankel (18) demonstrated its usefulness on the genital mucosa. But this was only the beginning. Before the end of the year 1884 numerous letters to the

editor had appeared on the successful use of cocaine as a local anesthetic in the Lancet and the lively interest was clearly demonstrated from many countries. Perhaps this is best illustrated from U.S.A. The New York ophthalmologist H. Knapp, editor of Archives of Ophthalmology, at the end of the year published an article there starting with the following words:

No modern remedy has been received with such general enthusiasm, none has been so rapidly popular, and scarcely any one has shown so extensive a field of useful application as cocaine, the local anesthetic introduced by Dr. C. Koller of Vienna.

Knapp then gave a translation of Koller's lecture before the Viennese Medical Society:

not only as an acknowledgment of a debt of gratitude we all owe to him, but also as an appropriate introduction.

Then followed the discussion of about 60 communications from American physicians who had employed cocaine anesthesia successfully. He also told about corresponding results from Germany, France, and Great Britain. In conclusion, he said that cocaine had been found useful in Ophthalmology, Otology, Rhinology, Laryngology, Pharyngology, Urology, Gynecology, and also in general Surgery (32).

The slow absorption of the cocaine solution into the interior of the eye from the conjunctival sac is a drawback and necessitated, during the early days of local anesthesia, preparation of the patient long before the operation. In a paper published in U.S.A. in 1892 (35), Koller wrote:

Very soon after my first communication I began using subconjunctival injection in squint operations, and during my time of assistantship to Professor Snellen performed many tenotomies and advancement of muscles, all with conjunctival applications of cocaine (after the conjunctiva had been anesthetized in the usual way). The operation can be made perfectly painless, and we have the double advantage of sparing the patient pain and not bringing discredit on a good drug.

It was of course to be expected, especially after von Anrep's experiments on himself, that the subcutaneous application of the drug would also soon be tried. The question was taken up by the young American surgeon W. S. Halsted (1852—1922), later called one of the leading lights of surgery in this country (49). It has also been said about him that "probably no one man has had a more profound effect upon American surgery" (29). Among his great contributions are also several concerning the use of cocaine in general surgery. For this he had to pay dearly, becoming innocently an addict to cocaine at a time when the risks of using it were unknown, and had great difficulties in getting free from his illness. His contributions are not well

enough known everywhere. In a letter to the editor of the New York Medical Journal R. J. Hall (25) described some cases in which he assisted Halsted where cocaine was successfully used subcutaneously or even to block certain nerves thus calling forth regional anesthesia. In a letter from Halsted to W. Osler (26) written many years later (1918) Halsted reports about his early successful use of cocaine anesthesia in major operations such as amputations, excision of joints etc., as well as the blocking of nerves and the effect of anemia in prolonging the anesthetic action. He also mentioned that he had demonstrated the application of cocaine in general surgery to Dr. A. Wolfier, Billroth's first assistant during a visit to Vienna in the autumn of 1885. According to Halsted, Wolfier had declared cocaine worthless in surgery, but after Halsted had shown him he became enthusiastic on the subject. In another letter of 1920 (27: 172-174) to C. L. Kells some further details are given. Halsted states there that he started to try cocaine—in collaboration with numerous students—one or two weeks at most, after the arrival of Koller's report. The valuable results of blocking the nerves for dental surgery were confirmed by E. H. Raymond (59). The influence of Halsted on Wolfier is mentioned by the latter (70) in the following terms:

In the beginning of November this year I had a visit from the practicing surgeon in New York Dr. Halsted who told me he had injected cocaine along the nerves concerned subcutaneously and thus obtained anesthesia. Therefore about a month ago I took up again the cocaine injections though I did not cure further about the course of the subcutaneous nerves.

He then mentioned that shortly before this A. Landreer (41) in Leipzig had published his experience of the successful use of cocaine injections subcutaneously in patients. Thus when E. Lesky states (44: 489) that it was Wolfier who made cocaine fruitful for surgery, this cannot be upheld, neither can her conclusion that cocaine anesthesia was the original work of the medical youth of Vienna, if not Koller *alone* is taken as the representative of the youth. Corning (11: 12) and Bier (4) share the merit of having shown the applicability of anesthesia by injection of local anesthetics into the cerebrospinal canal and several other methods of application have also been introduced.

There exists a remarkable parallelism between the situations after the introduction of general narcosis and of local anesthesia. This even involves certain personal experiences of the men who were primarily responsible for the great progress. In both cases it is true that their achievements did not contain a new revolutionary idea, since the fundamental observations had been done long before. Quoting once again the lecture by Clark (10) one must admit that

there is however a world of difference between a bright idea and a successful achievement and the main credit must always go to the man who possesses the energy, determination and skill to demonstrate the possibility of progress along some new path.

At the same time however controversial priority claims are more easily stated in such situations. Thus Professor M. J. Rossbach of the Pharmacological Institute of Würzburg where von Anrep carried out his work on cocaine protested that this had been ignored by Koller. In this however Rossbach was mistaken the error being due to the fact that he had not read the original of Koller's lecture but only a review of it (38 342—343).

As already mentioned (p. 16) Königstein spoke immediately after Koller before the Viennese medical Society on October 17 1884 giving a complete communication of his own experiments on the anesthetic effect of cocaine on the eye. He mentioned that on himself and other subjects he had observed that the conjunctiva and the cornea became anesthetised after the application of a one per cent cocaine solution that the pupil dilated moderately after about ten minutes and that accommodation decreased somewhat. He had tried cocaine with excellent results in extraction of foreign bodies from the cornea in incisions of chalazions etc. Pains from phlyctenular and pustular processes in the eye as well as vesicles from herpes zoster in the eye had also been treated with good results. During the days immediately before the meeting he had extracted an eye from a dog under cocaine without any sign of pain. Königstein however did not mention Koller's priority although it had been known for more than a month that he had made similar observations and had had them demonstrated in Heidelberg. Freud and Wagner Jauregg friends of both Koller and Königstein succeeded in persuading him to withdraw his claims on priority. When he published his own lecture in the *Wien med. Presse* in the issues of October 19 and 26 (39) he did not say anything about his omission but simply added the following sentences at the end of the paper:

"It remains to define my relations to the communication by Dr. Koller on September 15 at the Congress in Heidelberg and with regard to the fact that the complete anesthesia of the cornea and conjunctiva from cocaine were then for the first time pronounced I started my investigations independently of Mr. Koller but had at the time of his communication not arrived at a definite judgment regarding the anesthesia from coca also observed by myself. My further experiments soon gave me confirmation of Koller's statements though other observations mentioned here were absolutely independent."

One can well understand that Jones in his well known biography on Freud (31 95) noted that Königstein made his statement reluctantly. Jones' assertion is in fact an understatement and ought to be confronted with Koller's

statement (38 341) that Königstein when he heard that Koller had declared cocaine a perfect anesthetic for the eye operations said that he was mistaken. In this connection it seems of special interest that Königstein in his article (39) mentions that in one case he had obtained a cocaine solution which was without any effect. In this case the pharmacist had added too much acid in order to quicken the dissolving of the substance, and Königstein supposed that the irritating effect of the acid counteracted the anesthetic one of the cocaine. To-day it is of course well known that the real explanation is different. It is the free base cocaine that is efficient owing to its lipid solubility in contrast to the salts which are necessary for the absorption. But if the reaction is acid the dissociation becomes reduced and the effect will more or less disappear. It can under these circumstances not be excluded that Königstein after his negative result was more or less directly stimulated by Koller's success.

Jones has mentioned the intervention by Freud and Wagner Jauregg concluding in the following terms: 'As we shall see Koller did not reciprocate Freud's chivalrous behaviour' (31 95). It is a little difficult to find it chivalrous by Freud and Wagner Jauregg that they reacted against Königstein's attitude. Freud had asked him to try cocaine 'to alleviate the pain of certain eye complaints, such as trachoma and iritis' (31, 95) but without any idea that it could be successfully used in operations and his review on this point was as we have seen especially inadequate. He became as stated in a letter from him to Koller (38 342)

ashamed at the fact that in his published paper there is no mention of your name and I don't know how to explain it in view of my knowledge of him in other respects but I hope you will postpone taking any steps until I have talked to him and that you will after that create a situation in which he can retract.

To this might be added that Wagner Jauregg who knew Stricker's laboratory well also intervened. He was already known in those days as upright, firm-minded and fearless.

After reading the sentence quoted from Jones it is with some interest that one goes to his motivation and I quote his criticism in full (31 96). After mentioning that Koller emigrated to U.S.A. and had

a successful career (!) there

he said

But even at the beginning of his achievement he committed a symptomatic error which indicated some disturbance in his personality that came to open expression in later years. When publishing the paper he had read in Vienna in October 1884 he quoted Freud's monograph as dating from August instead of

July giving thus the impression that his work was simultaneous with Freud's and not after it. Both Freud and Obersteiner noticed this slip and corrected it in subsequent publications. As time went on Koller presented the discrepancy in still grosser terms even asserting that Freud's monograph appeared a whole year after his own discovery which was therefore made quite independently of anything Freud had ever done.

Perhaps we may correlate this curious behaviour with the fact that in hospital days Freud had treated him privately for a neurotic affection—negative transferences—as they are called—often endure.

It is certainly rare to meet in scientific literature such grave accusations with so little factual foundation. In the following all points concerned will be taken up and their real value will be illustrated. Jones starting point is that in quoting Freud's monograph (incomplete review would have been more exact) as dating from August instead of July Koller gives a certain impression. But in his Vienna lecture he explicitly emphasized that the review and the therapeutic results of Freud had called the attention of the Viennese doctors to cocaine. This is also quoted by Jones (31-95). How can it then be possible that Koller's work should have been simultaneous with that of Freud? Every careful reader will understand that Koller had not the least desire to deny that Freud's work had an *indirect* influence on his own. Both Freud and Obersteiner noticed the slip and corrected it in subsequent publications. Jones adds in foot note 1) to his paper of January 31 1885 (20) Freud mentions the date of his publication of the review adding in brackets (not August as often incorrectly cited) without however especially calling attention to Koller or to anybody else. Obersteiner (56) on the other hand gives the correct dating but without in any way complaining of errors from others—it is a pure guess that he tried to correct the slip. An illustration that Koller is not alone with regard to the slip is given by Königstein who naturally acknowledged that he had been directly inspired by Freud and made exactly the same mistake as Koller without however being accused of a symptomatic error. It seems probable that a common factor is responsible for the error e.g. that the reprints of the review became available or were sent to the friends of Freud rather late. The original article contained neither date of reception nor of publication. Furthermore Koller in his preliminary report to Heidelberg stated that he had performed his experiments the last weeks of August or even the beginning of September. Without a shred of evidence Jones a little earlier (31-94) tells us that Koller read Freud's essay when it appeared in July. When continuing his discussion of what he calls the discrepancy of Koller Jones gives as reference Koller's paper of 1935 (37). From this brief communication which is an addition to the paper published



interest

At this time I was asked by Freud to undertake together with him a series of experiments in order to test the effect of cocaine on muscular strength and fatigue. These experiments gave no particular results.

Later in the article he points out that

Moreno and I must admit that Freud had both pointed out that cocaine might be of practical use in surgery.

The paper, however, did not say in one word of what Jones has charged Koller with. Thus the unfortunate situation has arisen that the man who founded a group of people against another person on a simple mistake in the publication of a letter (instead of July) himself when referring to another paper gives no indication of publication nor even the journal correctly. I have been able to find the letter obviously meant by Jones. It is a letter from the 83 year old Koller to Dr M G Seelig (66) which the latter published with Koller's permission. In this letter Koller mentioned that he had greatly appreciated an article by Seelig about the progress in medicine during the last hundred years. He then wrote

Coming to the dealing with anesthesia I could not help being pained by your adopting the incorrect statements not of Freud himself but of his admirers and hangers on that Freud had anything to do with starting cocaine as a local anesthetic in surgery.

There followed a concise and correct report on the main steps of the historical development. Further on he wrote

Freud in his Autobiography regrets the fact that he passed by this great opportunity and gives full credit to me. I find fault with him only in that he failed to call off his over-enthusiastic admirers for giving credit to him for what he did not have any right to.

The facts are that Freud did not have anything whatever to do with cocaine anesthesia nor did he write a single word about work on cocaine in 1885 (whereas my work dates from 1884) that had not been done better and more scientifically by Anrep in 1879. Historical untruths are very difficult to destroy.

In a P S Koller mentions his article of 1935 (37) which

I published purposely in a Viennese medical weekly so that it would surely come under Freud's eye. I thought it might make him come and disavow the claims of his admirers that he had instigated my work on cocaine as a local anesthetic. However it had no such effect. I was on friendly terms with Freud and did not wish to accuse him directly of dishonesty. However his expression in his Autobiography that he had told me all of cocaine was so ambiguous as to cause all this untrue representation.

As these quotations clearly show it is not at all a question of Freud's review or monograph—about its indirect importance for stimulating people's interest in cocaine Koller had spoken several times before—but of Freud's own experimental studies published in some papers in 1885 the first and most interesting on January 31 1885 (20) i.e. several months after Koller's revolutionary paper. Jones himself has pointed out the rather poor quality of this contribution by Freud at the same time making the most of it (31 101). By substituting the year 1884 for 1885 and by introducing the word monograph Jones has tried to substantiate his remarkable interpretation. It is difficult to say whether Jones' mode of procedure should be characterized as the outcome of want of understanding or of carelessness but it is obvious that it ought not to belong in a biography with scientific claims. Jones finishes his criticism with a discreet suggestion that Koller's behaviour might be the consequence of an affection for which Freud had treated him in earlier days. As seen before (p. 7) Freud in a letter to Koller, had pointed out that he had been suffering from neurasthenia in contrast to what Freud had said before. Probably the majority of us would like Koller become neurasthenic if we were in danger of being deprived of the glory evoked by a great discovery and at the same time having the feeling of personal persecution. In his book Jones also mentions that Knapp the well known ophthalmologist when meeting Freud greeted him as the man who had introduced cocaine to the world and congratulated him on the achievement (31 103). This remark highly exaggerated as it is does of course not imply that Freud had given the inspiration to the use of cocaine as an anesthetic in surgery. On this point Knapp had as quoted above expressed in print the highest praise of Koller's work. This may have been unknown to Jones. In conclusion one finds that the criticism by Jones does not cast any shadow on Koller. Unfortunately the same cannot be said about Jones and the author has sometimes wondered whether the art of psychoanalysis could not with profit be applied to find an explanation for the numerous mistakes he has made.

There can be no doubt whatever about the priority of Koller with regard to the introduction of cocaine in surgery. Furthermore Freud himself in his paper (20) of January 31 1885 at a time when the whole story was fresh has written

While Dr. L. Königstein at my suggestion undertook to test the action of cocaine in alleviating pain and restricting secretions in pathological eye conditions Dr. Carl Koller quite independently happened upon the felicitous idea of inducing complete anesthesia of the cornea and conjunctiva by means of cocaine whose power to numb mucous membrane had been long known.

In spite of all the facts however the conception of a direct influence from

Freud on the initiative of Koller has still often been maintained e.g. in the monograph by Lofgren about xyllocaine or lidocaine (48) 1948 in Bonica's great handbook (6) from 1953 and even in the recent work by Faulconer and Keys (17) in 1965 in spite of the fact that they quote in full Koller's letter to Seelig (p. 771). A great responsibility in this connection rests on Jones whose erroneous presentation appeared many years after the death of Koller and consequently could not be answered by him. It is indeed high time that the real situation was made clear.

As mentioned before Koller has emphasized that Freud's work on cocaine focussed attention to the substance. Lebensohn (43) has characterized this *indirect* influence in the following words after having mentioned the unsuccessful results of Freud's experiments in order to cure morphine addiction with the aid of cocaine:

Curiously just as ether anesthesia was derived from the bizarre notions of Beddoes on pneumatic therapy so local anesthesia was derived from this mis-conception of Freud.

It is not astonishing that Freud personally regretted that he himself had missed the opportunity of making the great discovery that fell on Koller though it is probably true as pointed out by Jones (31-91) that it is not altogether likely that Freud even with more time at his disposal, would have thought of the surgical application one foreign to his interests. In fact scientific creative work usually demands concentration about a more or less circumscribed problem with intentional elimination of disturbing complications. As a recompense one will often find consolation in the conviction of having found something that others have passed over. Freud thought that he himself was dealing with a great discovery in the treatment of morphine addiction.

The parallelism between the two great discoveries of general narcosis and local anesthesia extends also to the unusually rapid acceptance of the new possibilities as well as to the insight that the methods are linked with certain dangers and have made new efforts necessary to build further on the fundamental discoveries. To this it must be added that the men who were mainly responsible for the introduction of the new methods into practical medicine met with unpleasant personal hardships. As is well known among the pioneers of general narcosis Morton died impoverished and embittered at the failure to obtain the reward that was discussed by the Congress of the USA during 16 years whereas Wells committed suicide and Jackson ended his days in a mental asylum. Only Long passed his life in relative tranquillity but he did nothing to make the discovery known to humanity as a whole but kept it to

himself. The fate of Koller was also greatly influenced by his discovery. In his letters to Nordenson (46) he complains of continuous depressing hostilities in Vienna obviously to a certain degree due to the prevailing anti-Semitism there. In the beginning of January 1885 this found expression in an episode at the General Hospital. Koller was in charge of the Admitting Room when a man with a seriously injured finger was brought in. He saw that there was immediate danger of gangrene owing to the application of a tight rubber bandage around the finger. A student from Billroth's surgical clinic asked that the patient be designed to this. Koller made a note of his wish and then cut the bandage whereupon the other doctor called him "Impudent Jew" or according to Freud in a letter to his fiancée (38-318) "Jewish Swine". Freud continued:

Now you must try to imagine the kind of atmosphere we live in here: the general bitterness — in short we would all have reacted just as Koller did by hitting the man in the face. The man rushed off, denounced Koller to the director who however called him down and categorically took Koller's side — — — But since they both are reserve officers he is obliged to challenge Koller to a duel and at this very moment they are fighting with sabres under rather severe conditions.

At the end of the letter Freud added:

Our friend is quite unharmed and his opponent got two deep gashes. We are all delighted & proud day for day.

For Koller however the result increased his difficulties since he must leave the country, duels being forbidden and thus also his hopes of obtaining the position of assistant at the Eye Clinic to which he otherwise would be very well qualified and an academic career for which he probably had hoped also became excluded. It was during this period that Koller became depressed. It was also then that he met Nordenson and obtained his help in the continuation of his training within ophthalmology and at last emigrated to New York. During his long stay there he received many distinctions, such as several gold medals, honorary memberships inter alia in Wiener medizinische Gesellschaft (Viennese Medical Society) 1930. He was several times proposed for the Nobel Prize in Physiology or Medicine. His discovery was undoubtedly prize-worthy but it was too old and had been generally accepted the same year and thus could not be considered at a time when the medical faculty tried carefully to follow the statutes in awarding the prize. Their old discoveries only in case their importance had been acknowledged recently.

It is not possible here to describe the continued development of cocaine anesthesia. The general toxicity of cocaine has caused numerous deaths and attempts have been made to avoid them by using very dilute solutions.

was emphasized at an early stage by Halsted (27 178) but the practical application became mainly the work of the Frenchman P. Reclus (60) and the German C. L. Schleich (63). Halsted has pointed out (27 519) that after incising the anesthetized skin the underlying parts

were comparatively insensitive to handling and for the most part even to cutting. The accidental cutting or crushing of nerves caused the most exquisite pain and we noticed that the nerve supply of the blood vessels is so abundant that the moving or clasp- ing of even very small bleeding points usually started a cry of some sort of remonstrance from the patient and now after many years of experience with cocaine we interpret an unexpected moan as signifying an insult to some small unseen blood vessel or nerve.

Among the numerous new preparations introduced in the place of cocaine procaine (novocaine) has been especially valuable. It was introduced by the chemist Einhorn (14) and the surgeon H. Braun (7—9). The latter performed a comparison with other substances indicated a simple method for the assay and introduced the addition of epinephrine to such substances as—like procaine but in contrast to cocaine—cause dilation of vessels. Together with Halsted Braun is certainly the clinician who has made the greatest contributions to the field. Lastly, it should be mentioned that the preparation which is at present most used for local anesthesia, namely the Swedish discovered xylocaine (lidocaine) originated from the observation of the numbing effect on the tongue by a substance (15) by H. Erdtman which gave rise to a large number of derivatives before the new remedy took form. Thus the effect on the tongue this time was not overlooked but like Koller one understood to exploit the observation.

On the ground once laid by Carl Koller an important branch of medical science and practical work has thus been successfully developed. It is the old story of a young scientist who has been fascinated by an idea and thanks to this and his special training has been ready to take the opportunity when it has suddenly presented itself. But it is also an illustration of the famous pronouncement by Pasteur that chance only favors those whose minds have been prepared. At the same time Koller had the invaluable qualities of being true, reliable and fearless. (5)

## References

- 1 Adamkiewicz A (1886) Die Diffusions-elektrode *Neurol Zbl* 5 219—222
- 2 Anrep H von (1880) Ueber die physiologische Wirkung des Cocain. *Pfl Arch ges Physiol* 21 38—77
- 3 Berg F (1965) *Oftalmologin i Sverige under 1800 talet*. Almqvist och Wiksell Uppsala (Swedish) pp 321 Quot from ■ 272
- 4 Bier A (1899) Versuche über Cocainisierung des Rückenmarks *Dtsch Z Chir* 51 361—369
- 5 Bloom S (1944) Carl Koller M D 1857—1944 *Arch Ophthalmol* 31 344—345
- 6 Bonica J J (1953) *The Management of Pain*. Lea and Febiger, Philadelphia pp 1533
- 7 Braun H (1905) *Die Lokalanästhesie ihre wissenschaftlichen Grundlagen und praktische Anwendung*. J A Barth Leipzig pp 436
- 8 Braun H (1925) *Die örtliche Betäubung ihre wissenschaftlichen Grundlagen und praktische Anwendung*. J A Barth Leipzig pp 511
- 9 Braun H und Lärén A (1933) *Die örtliche Betäubung* 8 Aufl J A Barth Leipzig pp 511
- 10 Clark A J (1938) Aspects of the History of Anaesthetics *Brit med J* II 1029—1034
- 11 Corning J L (1885) Spinal Anaesthesia and local Medication of the Cord *N Y med J* 42 493—485
- 12 Corning J L (1888) A further Contribution on local Medication of the spinal Cord *Med Rec (N Y)* 33 291—293
- 13 Dawbarn R H M (1891) Water as a local anesthetic Its discovery American and not German *Med Rec (N Y)* 40 613
- 14 Einhorn A und Uhlfelder E (1909) Ueber den p-Aminobenzoesäure diäthyl amino und Piperidoäthylester *Justus Liebigs Ann Chem* 371 131—142
- 15 Euler H von und Erdman H (1935) Ueber Gramin aus schwedischen Gersten sippen *Justus Liebigs Ann Chem* 520 1—10
- 16 Fabritius W (1964) 80 Jahre seit der Einführung der Cocainanästhesie in der Ophthalmologie durch Carl Koller *Communic ex Bibliotheca historiae medicae Hung* 32 137—144
- 17 Faulconer A and Keys T E (1965) *Foundations of Anesthesiology* 2 vol C G Thomas Springfield pp XL + 1337 + I.
- 18 Frankel E (1884) Über Cocain als Mittel zur Anästhesierung der Genitalschleim haut *Sten med Wsch* 34 1535
- 19 Freud S (1884) Ueber Coca *Cbl ges Ther* 2 289—314 19 b In English Translation in S Freud (1963) *The Coca Papers* 1—26 Dunquin Press Vienna and Zurich

was emphasized at an early stage by Halsted (27 178) but the practical application became mainly the work of the Frenchman P Reclus (60) and the German C L Schleich (63) Halsted has pointed out (27 519) that after incising the anesthetized skin the underlying parts

were comparatively insensitive to handling and for the most part even to cutting The accidental cutting or crushing of nerves caused the most exquisite pain and we noticed that the nerve supply of the blood vessels is so abundant that the severing or clamping of even very small bleeding points usually started a cry of some sort of remonstrance from the patient and now after many years of experience with cocaine we interpret an unexpected moan as signifying an insult to some small unseen blood vessel or nerve

Among the numerous new preparations introduced in the place of cocaine procaine (novocaine) has been especially valuable It was introduced by the chemist Einhorn (14) and the surgeon H Braun (7—9) The latter performed a comparison with other substances indicated a simple method for the assay and introduced the addition of epinephrine to such substances as—like procaine but in contrast to cocaine—cause dilation of vessels Together with Halsted Braun is certainly the clinician who has made the greatest contributions to the field Lastly, it should be mentioned that the preparation which is at present most used for local anesthesia namely the Swedish discovered xlocaine (lidocaine) originated from the observation of the numbing effect on the tongue by a substance (15) by H Erdtman which gave rise to a large number of derivatives before the new remedy took form Thus the effect on the tongue this time was not overlooked but like Koller one understood to exploit the observation

On the ground once laid by Carl Koller an important branch of medical science and practical work has thus been successfully developed It is the old story of a young scientist who has been fascinated by an idea and thanks to this and his special training has been ready to take the opportunity when it has suddenly presented itself But it is also an illustration of the famous pronouncement by Pasteur that chance only favors those whose minds have been prepared At the same time Koller had the invaluable qualities of being true reliable and fearless (5)

- 41 Landerer A (1885) Lokale Anästhesie mit subkutanen Cocaininjektionen *Cbl Chir* 12 841—842
- 42 Larrey D J (1817) *Mémoires de Chirurgie militaire et Campagnes III* J Smith Paris pp 500
- 43 Lebensohn J F (1934) The Semicentenary of local Anesthesia. *Amer J Ophthal* 17 949—952
- 44 Lesky E (1965) *Die Wiener medizinische Schule im 19 Jahrhundert* H Bohlaus Nachf Graz Köln pp 660
- 45 Liljestrand G (1962) The Prizes in Physiology or Medicine Nobel the Man and his Pri et sec ed 35—343 Elsevier Amsterdam
- 46 Liljestrand G (1965) Briefe Carl Kollers an Erik Nordenson *Arch Gesch Med* 49 280—306
- 47 Liljestrand G (1965) The historical Development of local Anesthesia. Manuscript accepted for *Encyclopedia of Pharmacology and Therapeutics* vol Local Anesthetics Pergamon Press Oxford
- 48 Lofgren N (1948) *Studies in local Anesthetics* *Ethylchloride a new synthetic Drug Thesis* I Haeggstrom Stockholm 150 pp Reprinted Worcester Mass 1958
- 49 Matas R (1947) William Stewart Halsted 1852—1927 An Appreciation *Surgical Papers by William Stewart Halsted 1853—1927* I XL—XLIII
- 50 Matas R. (1934) Local and Regional Anesthesia A Retrospect and Prospect *Amer J Surg* 60 189—198 362—379
- 51 Moreno y Maiz T (1868) *Recherches chimiques et physiologiques sur l'Erythroxylum Coca du Perou* Thèse de Paris pp 91
- 52 Niemann A (1860) *Ueber eine neue organische Base in den Cocablättern* Inaug diss Göttingen pp 57 A preliminary report was given by F Wohler (1860) *Ueber eine organische Base in der Coca* Justus Liebig's *Ann Chem* 114 213—217
- 53 Nordenson J W (1931) *Tal vid doktorspromotionen vid Kongl Universitetet i Uppsala lördagen den 30 maj* (Swedish)
- 54 Nordenson J W (1962) All ar Gullstrand (1862—1930) *Documenta ophthal* 16 283—337
- 55 Noyes H D (1884) The ophthalmologic Congress in Heidelberg *Med Rec* 26 417—418
- 56 Oberstörner H (1885) Zur intern n Anwendung des Cocains bei Neurosen und Parosien *Wiener med Presse* 26 1253—1257
- 57 Pare A (1664) *Les Œuvres* 12 ed Lyon I 306
- 58 Paschke H und Wagner J (1886) Ueber die durch Chloroform auf kataphoretischem Wege zu erzeugende Hautanästhesie (Adamkiewicz) *Neurol Zbl* 5 413—416
- 59 Raymond E H (1885) Hydrochlorate of Cocaine as a local Anesthetic in Dental Surgery Reproduced in *Surgical Papers by W S Halsted* (27) I 171—172
- 60 Reclus P (1895) *La Cocaine en Chirurgie* G Masson Paris pp 197
- 61 Richet A (1854) Anesthésie localisée *Gaz hop* (Paris) 27 251—252 263—264 267—268
- 62 Rusconi (1885) Il cloridato di cocaina in ginecologia. *Gaz. degli ospit* 6 1—3
- 63 Schleier C L (1894) *Schmerz lose Operationen Örtliche Betäubung mit indifferenten Flüssigkeiten* Springer Berlin pp 256 (5th ed 1906 pp 331)





ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 300

STUDIES ON THE BIOSYNTHETIC  
PATHWAY OF POLYAMINES  
IN RAT LIVER

BY

JUHANI JÄNNE



ACTA PHYSIOLOGICA SCANDINAVICA

— VOLUME 92, SUPPLEMENT 1, 1967 —

STUDIES ON THE BIOSYNTHETIC  
PATHWAY OF POLYAMINES  
IN RAT LIVER

BY

JUHANI JÄNNE

HELSINKI 1967



ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 300

FROM THE DEPARTMENT OF MEDICAL CHEMISTRY  
UNIVERSITY OF HELSINKI FINLAND

STUDIES ON THE BIOSYNTHETIC  
PATHWAY OF POLYAMINES  
IN RAT LIVER

BY

JUHANI JÄNNE

HELSINKI 1967



To my wife





## Preface

This work was carried out in the Department of Medical Chemistry University of Helsinki. I am greatly indebted to my honoured chief Professor JOHAN JÄRNEFELT M.D. for his interest in my work and for placing all the facilities of his department at my disposal.

I wish to express my deepest gratitude to my teacher and friend Dr AARNE RAINA M.D. for his continued support in the course of each stage of this study. His interest and expert advice have been of inestimable help during my work.

Further, I wish to express my gratitude to Docent NILS ERIK SARIS Ph.D. and Docent ELJA PITKÄNEN M.D. for their criticism of the manuscript.

I am grateful to my colleagues in the Department of Medical Chemistry. In particular, my thanks are due to my friend Dr MARTTI SIIMES for his intellectual and technical assistance during this work, to Docent NIELS RÄIHA M.D. for many stimulating discussions and to Mr TIMO TELARANTA B.M. and Mr OLLI JÄNNE B.M. for their advice at various stages of my work.

I am very grateful to Mrs SIRKKA KANERVA for the skilful laboratory assistance which made my work possible.

Miss ANN MATHESON M.A. has corrected the English text. I thank her for her collaboration and interest in my work.

Helsinki May 1967

JUHANI JÄNNE



# CONTENTS

INTRODUCTION	7
REVIEW OF THE LITERATURE	8
Occurrence	11
Biosynthesis	11
Degradation	14
Some aspects of the possible physiological role of putrescine and polyamines	14
OUTLINE OF PRESENT STUDY	19
MATERIAL AND METHODS	20
Experimental animals	20
<i>In vitro</i> techniques	20
<i>In vivo</i> techniques	21
Analytical methods	21
RESULTS	21
I Occurrence of putrescine and its conversion into polyamines in rat liver	21
Concentration of putrescine in liver at different ages	24
Conversion of putrescine into polyamines in rat liver	25
Fate of the radioactivity from exogenously administered $^{14}\text{C}$ putrescine	26
Biosynthesis of spermidine from putrescine in liver homogenates	29
The early effect of ethionine on the biosynthesis of spermidine	31
Concluding remarks	31
II Biosynthesis of putrescine and polyamines in regenerating rat liver	35
The contents of putrescine and polyamines in rat liver after partial hepatectomy	35
Synthesis of putrescine from $^{14}\text{C}$ -ornithine in regenerating liver	37
Synthesis of spermidine from $^{14}\text{C}$ putrescine in regenerating liver	39
Spermidine synthesis from $^{14}\text{C}$ putrescine in liver homogenates obtained from partially hepatectomized rats	42
Incorporation of radioactivity from $^{14}\text{C}$ arginine into putrescine spermidine and liver total protein after partial hepatectomy	43
Effect of putrescine concentration on the incorporation of radioactivity from $^{14}\text{C}$ methionine into spermidine in liver homogenates	50
Concluding remarks	49
III Effect of growth hormone on putrescine and polyamine biosynthesis in rat liver	50
Hepatic putrescine and polyamine concentrations after a single injection of growth hormone into normal rats	51
Effect of growth hormone on putrescine synthesis from $^{14}\text{C}$ ornithine	54
Biosynthesis of polyamines from $^{14}\text{C}$ putrescine after growth hormone treatment	53
Incorporation of radioactivity from $^{14}\text{C}$ methionine into liver spermidine spermine and total protein after growth hormone treatment	56
Concluding remarks	5
DISCUSSION	54
SUMMARY	63
REFERENCES	67



## Introduction

Putrescine spermidine and spermine are aliphatic amines occurring in almost all living cells. The wide distribution of these compounds has been known for years but the significance of their presence has not been established.

Studies concerning the metabolism and effects of diamines and polyamines on cellular level have for the most part been carried out on microorganisms and the knowledge we have of their association with the intermediary metabolism of the mammalian tissues is rather meagre.

Putrescine spermidine and spermine have unquestionably many effects on various cellular and subcellular components *in vitro* and most of these effects can be explained on the basis of their polycationic nature i.e. the stabilizing effects they exert on nucleic acids are in most cases comparable with those observed with higher concentrations of  $Mg^{++}$  or  $Ca^{++}$ .

In recent years increasing evidence has been accumulated indicating that these compounds might also play an active role in many fundamental biological phenomena in mammalian tissues. The changes taking place in the polyamine pattern during the growth processes of an animal tissue or organ whether developmental or regenerative makes it justifiable to assume that they are in some way related to cell multiplication and tissue growth.

# Review of the literature

**Structure** The diamine putrescine  $\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}_2$  is the decarboxylated derivative of the amino acid ornithine. Spermidine  $\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}_2$  also contains in its molecule in addition to the putrescine fragment a propylamine moiety. Spermine  $\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}_2$  contains one putrescine fragment and two propylamine moieties.

## OCCURRENCE

**Microorganisms** Most of the studies dealing with the metabolism of putrescine, spermidine and spermine have been carried out on microorganisms. *Escherichia coli*, a bacterium which has been studied in the greatest detail, contains when grown in minimal medium 15  $\mu\text{moles}$  of putrescine and 15  $\mu\text{moles}$  of spermidine per g wet weight (TABOR and TABOR 1964). Part of the putrescine and spermidine in this bacterium occurs in the form of acetylated derivatives (DUBIN and ROSENTHAL 1960). The concentrations of these amines, however, vary markedly with the cultural conditions, i.e. with pH (TABOR, ROSENTHAL and TABOR 1958). Comparable concentrations of putrescine and spermidine have been obtained from various other bacteria (TABOR and TABOR 1964). There are, however, wide variations in the amounts found in different microorganisms. In general, it can be concluded that only gram-negative bacteria contain putrescine and spermidine in the above-mentioned concentrations. Most gram-positive bacteria neither store nor excrete putrescine (HERBST, WEAVER and KEISTER 1958). In contrast to the considerably high concentrations of putrescine and spermidine, most microorganisms contain little or no spermine (TABOR and TABOR 1964).

In addition to the bacterium itself, the bacterial viruses also contain putrescine and spermidine in high concentrations. The neutralization of one third to one half of the deoxyribonucleic acid phosphate in the bacteriophage can be accounted for by putrescine and spermidine. The content of these amines, however, varies considerably with the type of the virus and in the first place with the putrescine and spermidine content of the host bacterium. Further, putrescine and spermidine can be replaced by other cationic compounds, suggesting that these amines may act as unspecific cations neutralizing negatively charged phosphate groups of the phage deoxyribonucleic acid (AMES, DUBIN and ROSENTHAL 1958; AMES and DUBIN 1960).

Putrescine and spermidine, in addition to bacteria and viruses, are naturally occurring components in many plants and plant alkaloids. The distribution of diamines and polyamines in the vegetable world is reviewed in detail by TABOR and TABOR (1964).

*Animal tissues* - The first report of the occurrence of polyamines in animal tissues can be found in Lewenhoeck's letter to the Royal Society (see ref TABOR and TABOR 1964) The crystals he saw in human semen were probably spermine phosphate

One of the first systematic studies on the occurrence of spermine in various human tissues was reported by HÄMÄLÄINEN (1947) He observed that almost all human tissues contained spermine The highest concentrations of this polyamine were found in prostate pancreas and bone marrow The results of this study were generally in agreement with those earlier reported by DUDLEY and ROSENHEIM (1925) WREDE (1926) and HARRISON (1933)

The lack of adequate analytical methods at that stage prevented the study of small tissue samples and small laboratory animals The introduction of a sensitive method by ROSENTHAL and TABOR (1956) made it possible to determine the polyamine content in various tissues and organs of rats mice guinea pigs and rabbits This study included only a few tissues and organs but some general conclusions could be drawn First in most of the tissues studied the molar concentration of spermidine exceeded that of spermine Secondly there were great variations in the polyamine content in different tissues of the same animal e.g. rat pancreas contained 8.6  $\mu$ moles of spermidine and 1.0  $\mu$ mole of spermine per g wet weight rat testis however only 0.4 and 0.5  $\mu$ moles respectively Rat liver and in particular rat prostate contained polyamines in high concentrations The amounts of polyamines in some experimental tumours varied but generally a high molar ratio of spermidine to spermine could be observed

The polyamines spermidine and spermine are also found in animal blood (ROSENTHAL and TABOR 1956 RAINA 1962a SHIMIZU KAKIMOTO and SANO 1965a) As stated by ROSENTHAL and TABOR (1956) spermine in the blood occurs in the formed elements and none is found in the plasma This fact which also holds true for spermidine was later confirmed by RAINA (1962a) using human blood SHIMIZU KAKIMOTO and SANO (1965a) assumed that spermine occurs in or around the cell nucleus This idea was based on the findings indicating that the concentration of spermine was considerably high in the blood of non mammals whose erythrocytes have nuclei

The association of diamines and polyamines with growth processes is an interesting feature in the metabolism of these compounds in animal tissues None of diamines or polyamines could be detected in unincubated eggs (RAINA 1963 CALDARERA BARBIROLI and MORUZZI 1965) On the other hand the concentrations of spermidine and spermine in the embryos increased sharply during incubation reaching two typical peaks during the 2nd - 3rd and 15th - 16th days of incubation (RAINA 1963) In addition to the polyamines the diamines putrescine and cadaverine (decarboxylated derivative of lysine) also showed similar changes during the development of the chick embryo (CALDARERA BARBIROLI and MORUZZI 1965) These changes in the polyamine pattern correlated very well with those taking place in other areas of embryonic metabolism e.g. the concentrations of nucleic acids showed parallel behaviour at each stage of the development of the chick embryo (CALDARERA BARBIROLI and MORUZZI 1965)



Based on the above investigations it seems that the age of an embryo or animal is an important factor affecting the metabolism of diamines and polyamines. This fact was later confirmed by determining the concentrations of polyamines in various rat tissues in relation to age (JÄNNE KAINA and SIIMES 1961). Again great variations between different tissues of the same rat was observed but some generalization could be made. Metabolically active and cell rich tissues such as the liver and thymus of a young animal contained much more spermidine than spermine. Further in all the tissues studied the concentration of spermidine decreased without exception with increasing age. The decrease was in general most marked during the first month of life. The changes in the concentration of spermine were considerably smaller but an increase in the concentration of this polyamine was observed mostly during the first month. The changes in polyamine content with increasing age were reflected by the molar ratio of spermidine to spermine. In the liver for instance this ratio was 4.52 at birth then decreased steadily being only 0.84 9 months after birth. This decrease in spermidine and usually a slight increase in spermine was a uniform feature in all the tissues studied. Similar results were later reported by SHIMIZU KAKIMOTO and SANO (1965b) using some mice tissues. A correlation between spermidine and deoxyribonucleic acid was also observed.

In contrast to the investigations dealing with the occurrence of spermidine and spermine in animal tissues there are only a few studies concerning the distribution of the diamine putrescine in the animal world. This diamine is in fact the main aliphatic amine in bacteria and bacterial viruses and as will be seen later acts as a quite effective precursor of spermidine biosynthesis in animal tissues too. There are reports of the isolation of diamines from animal material but these were in most cases cadavers undergoing bacterial decomposition (GUGGENHEIM 1951). The concentration of putrescine found in fresh tissues of some mammalian species has been reported to be very low as compared with the concentrations of spermidine and spermine. According to WEAVER and HERBST (1958) the concentration of putrescine in liver of several species was only about 1/50 of the spermidine and spermine concentration. The concentration of putrescine compared with that of polyamines was still lower in the porcine and bovine pancreas and not a trace of this diamine can be detected in bovine liver according to FISCHER and BOHN (1957).

Considerable amounts of the diamines putrescine and cadaverine however were found in the developing chick embryo. During the embryonic development the concentrations of these showed peaks preceding those of spermidine and spermine but thereafter decreased steadily until hatching. Only trace amounts of these compounds could be found in 4 day old chicks (CALDARERA BARBIROLI and MORUZZI 1965). Some evidence indicating the presence of putrescine in the chick embryo has also been obtained by RAINA (1963).

*Intracellular distribution of putrescine, spermidine and spermine.* Putrescine, spermidine and spermine as cations have a high affinity for various acidic compounds occurring within bacterial or animal cells. This phenomenon is precisely very interesting in living cells but unfortunately it also complicates the determination of the intracellular distribution of these amines since after disintegration of the cell secondary redistribution can take place. The diamines

and polyamines are thus after homogenization of the tissue rapidly taken up by various particulate fractions of the cell (TABOR and TABOR 1964)

COHEN and LICHTENSTEIN (1960) found that the ribosomal fraction of *E. coli* contained putrescine and spermidine in amounts equivalent to approximately 15 per cent of their total content in this bacterium. Further they concluded that ribosomal putrescine which was the main amine and spermidine occurred in a nonexchangeable form and were not derived from the extracting medium. However according to TABOR and KELLOG (1967) the polyamine content of *E. coli* ribosomes is dependent on the nature and concentration of the ions in the medium used for their preparation.

The molar amounts of putrescine and spermidine have been calculated to be sufficient to neutralize about 8 per cent of the ribosomal ribonucleic acid phosphate in *E. coli* (COHEN and LICHTENSTEIN 1960). According to ZILLIG, KRONE and ALBERS (1959) the amine content of *E. coli* mainly putrescine and cadaverine in this study could neutralize at least a third of the acidic groups of the ribosomal ribonucleic acid. The presence of the diamine cadaverine in the ribosomal particles of *E. coli* has also been stated by SPAHR (1962). The total diamine and polyamine content amounted to 0.4 per cent of the dry ribosome and the amines were found approximately in a molar ratio putrescine/spermidine/cadaverine 3:2:1. Only traces of spermine were detected.

If the polyamines in *E. coli* ribosomes at least to some extent are non-exchangeable, the ribosomes isolated from guinea pig pancreas rapidly take up labelled spermidine from the medium (SIEKEVITZ and PALADE 1964). This also held true for liver homogenate in which exogenously added labelled polyamines were quickly taken up by particulate fractions of the disintegrated cell (TABOR and TABOR 1964).

The subcellular distribution of the polyamines spermidine and spermine in rat liver has recently been studied by RAINA and TELARANTA (1967). About 60 per cent of the total cell polyamines was recovered from the microsomal fraction at a low ionic medium and the percentage distribution of spermidine and spermine was very close to that of ribonucleic acid in isolated subcellular fractions. With increasing ionic strength, especially in the case of divalent cations, the polyamines were partly released from the microsomes into the medium. The added labelled polyamines were taken up by particles and again released by increasing the ionic strength.

In spite of the possibility of redistribution of polyamines after disintegration of a mammalian cell, it seems that in all probability the intracellular polyamines are bound to nucleic acids and according to SIEKEVITZ and PALADE (1964) as well as RAINA and TELARANTA (1967) the binding is due to a salt bond between the amine and ribonucleic acid phosphate.

## BIOSYNTHESIS

*In microorganisms* The first experiments demonstrating the biosynthesis of spermidine in *E. coli* and *Aspergillus nidulans* were carried out by TABOR, ROSENTHAL and TABOR (1956, 1957, 1958). They showed that the

four carbon moiety of the polyamine molecule was derived from putrescine  $^{14}\text{C}^{15}\text{N}$  putrescine was incorporated as a unit into spermidine molecule in growing cultures of *E. coli* and *A. nidulans*. Using an ornithine requiring mutant of *E. coli* they also showed that this amino acid could serve as a precursor for putrescine and spermidine synthesis.

GREENE (1957) demonstrated that the propylamine unit of spermidine molecule was derived from the amino acid methionine.

The biosynthesis of spermidine from putrescine and methionine has also been demonstrated with cell free extract of *E. coli* (TABOR ROSENTHAL and TABOR 1958).

As stated previously ornithine can act as a precursor of putrescine synthesis in *E. coli* indicating that one of the precursors of putrescine is a late intermediate of the arginine biosynthetic pathway. GALE (1940) demonstrated an induced ornithine decarboxylase in *E. coli* which in special culture conditions catalyzed the formation of putrescine. This enzyme however could not be responsible for the biosynthesis of large amounts of putrescine normally occurring in *E. coli* grown in minimal medium. Recently MORRIS and PARDEE (1965, 1966) have reported the presence of an other type of enzyme decarboxylating ornithine in *E. coli*. They called this enzyme a biosynthetic ornithine decarboxylase to distinguish it from the inducible enzyme described by GALE (1940). This biosynthetic enzyme differed from the latter in its pH optimum, heat stability and various other properties. The decarboxylation of ornithine to yield putrescine was also catalyzed in cell free preparations (MORRIS and PARDEE 1965, 1966).

Not only ornithine but also arginine was decarboxylated in cultures of *E. coli*. The decarboxylation of arginine serves as an other route for putrescine formation in *E. coli*. Again the presence of two distinct enzymes for the decarboxylation of arginine is obvious in this organism. Like ornithine decarboxylase there is an inducible (GALE 1940) and a biosynthetic (MORRIS and PARDEE 1966) arginine decarboxylase in *E. coli*. The conversion of arginine to putrescine involved two distinct reactions: decarboxylation yielding agmatine (decarboxylated arginine) and subsequent hydrolysis of the guanidino group of agmatine yielding putrescine and urea as final products (MORRIS and PARDEE 1966). It is unlikely that ornithine is an intermediate in this reaction since the presence of arginase activity in *E. coli* has never been reported (MORRIS and PARDEE 1966).

The formation of spermidine from putrescine and methionine involves first the conversion of methionine to S-adenosylmethionine. The mechanism for this methionine activation is that previously described for transmethylation (CANTONI and DURELL 1957). The formation of S-adenosylmethionine is followed by a subsequent decarboxylation of this compound. The propylamine moiety from the decarboxylated S-adenosylmethionine is then transferred to putrescine to yield spermidine (TABOR and TABOR 1964).

Putrescine is also incorporated into spermine (TABOR ROSENTHAL and TABOR 1958) suggesting the possible role of spermidine to act as a precursor of spermine biosynthesis.

The various steps involving spermidine biosynthesis in *E coli* are summarized in Fig 1

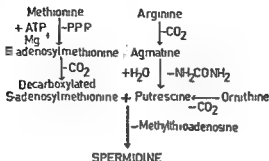


Fig 1 Spermidine synthesis in *E coli*

In addition to the synthesizing capacity of *E coli* this bacterium can also take up putrescine and polyamines from the medium. The amines are adsorbed by the bacterium as well as actively transported through a time and metabolic processes-dependent system which does not occur at 0°C and is markedly decreased by the omission of glucose (TABOR and TABOR 1966).

**In animal tissues** The first evidence indicating the biosynthesis of polyamines from putrescine in mammalian tissues was obtained by TABOR, FOSHANTAL and TABOR (1956). In a preliminary paper they reported a small incorporation of radioactivity from <sup>14</sup>C<sup>15</sup>N putrescine into spermidine in minced rat prostate.

RAINA (1962b, 1963) demonstrated that the known precursors of spermidine in bacteria i.e. putrescine, ornithine and methionine were also incorporated into spermidine and spermine in developing chick embryos. The radioactivity from <sup>14</sup>C putrescine or <sup>14</sup>C ornithine was rapidly incorporated into spermidine whereas a lag period of several hours was obvious before radioactive spermine could be isolated. On the other hand the incorporation of radioactivity from <sup>14</sup>C methionine into both polyamines occurred at almost equal rates.

Putrescine and methionine (RAINA 1964) as well as the amino acid arginine (JÄNNE and RAINA 1966) have been shown to serve as precursors of polyamines in various rat tissues. After the injection of putrescine into young rats the label was again found in the early phase mainly in spermidine whereas the label from methionine was transferred roughly equimolarly into both polyamines (RAINA 1964). These results suggested that in animal tissues spermidine acts as a precursor for spermine synthesis.

The role of spermidine in serving as a precursor for spermine formation in animal tissues has later been confirmed indirectly (RAINA, JÄNNE and SIIMES 1964) and recently directly (JÄNNE and RAINA 1966, SIIMES 1966, 1967).

Interestingly the converse reaction formation of spermidine from spermine has also been demonstrated as occurring in rat tissues (SIIMES 1967)

On the other hand after administration of uniformly labelled  $^{14}\text{C}$  glucose or  $^{14}\text{C}$  proline to chick embryos no radioactivity could be detected in the polyamines (RAINA 1963) Nor was the label from the uniformly labelled  $^{14}\text{C}$  glutamate incorporated into spermidine or spermine isolated from the rat liver (JÄNNE and RAINA 1966)

In addition to the preliminary paper by TABOR ROSENTHAL and TABOR (1956) the synthesis of polyamines has not been reported to occur in animal tissues *in vitro* Recently however the decarboxylation of ornithine spermidine synthesis from putrescine and the interconversion reactions between spermidine and spermine have been demonstrated in Ehrlich ascites cells *in vitro* (SIIMES and JÄNNE 1967)

Little is known of the distribution of putrescine and even less is known of its biosynthesis in animal tissues After administration of  $^{14}\text{C}$  ornithine to chick embryos RAINA (1963) could detect a weak ninhydrin positive fraction which contained radioactivity This fraction showed a mobility similar to putrescine in paper electrophoresis and paper chromatography The observation suggests that in animal tissues too the polyamines spermidine and spermine are synthesized via putrescine

## DEGRADATION

In addition to the above mentioned interconversion reactions between spermidine and spermine very little is known of the metabolic pathways involving the degradation of these compounds in animal tissues *in vivo* Some additional evidence suggesting the degradation of spermine to spermidine has however been obtained When spermine is injected parenterally into an animal a small part of the dose appears as spermidine in the urine On the other hand when spermidine is administered no spermine is excreted (ROSENTHAL and TABOR 1956)

Using  $^{14}\text{C}$  labelled polyamines SIIMES (1967) could demonstrate that spermidine and spermine are convertible into each other in rat tissues Further both polyamines were oxidized into carbon dioxide in the rat *in vivo*

The oxidation of diamines and polyamines in animal tissues by plasma amine oxidase (HIRSCH 1953a 1953b) as well as their oxidative and non oxidative degradation in microorganisms are reviewed in detail by TABOR and TABOR (1964)

## SOME ASPECTS OF THE POSSIBLE PHYSIOLOGICAL ROLE OF PUTRESCINE AND POLYAMINES

*In microorganisms* Many of the effects these amines exert on various cellular and subcellular components could be explained on the basis of their physico-chemical structure The diamines and polyamines are basic substances having a high affinity to the acidic groups of many natural compounds By

considering diamines and polyamines as polycations the stabilizing effects they exert on nucleic acids for instance can be understood. In general the effects are in many instances closely related to those observed with histones and protamines as well as divalent cations  $Mg^{++}$  and  $Ca^{++}$ .

One of the first signs suggesting the physiological importance of these compounds was that indicating their requirement for the growth of certain microorganisms: i.e. *Hemophilus parainfluenzae* (HERBST and SNELL 1949) and some strains of *Neisseria* (MARTIN PELCZAR and HANSEN 1954).

Various features of the effect of polyamines on nucleic acids have been reported *in vivo* and *in vitro*. This area of polyamine function however is also reviewed in detail by TABOR and TABOR (1964) but some of the latest investigations will be cited here.

The polyamines spermidine and spermine clearly increased the denaturation temperature of deoxyribonucleic acid obtained from *Bacillus subtilis* (TABOR 1961). Similar stabilizing effects by polyamines have also been observed on ribonucleic acid (MITRA and KAESBERG 1963, GOFDSTEIN 1966).

Polyamines also exert great influence on the reactions involving nucleic acids as substrates. Spermine inhibits ribonuclease enzyme in yeast cells (SCHLENK and DAINKO 1966). Spermidine and spermine inhibit the degradation of nucleic acids under various other conditions. These effects are however considered to be an indirect inhibition of nucleases based possibly on stabilization of the cells or subcellular particles by polyamines (TABOR and TABOR 1964).

Putrescine, spermidine, spermine and other related compounds stimulated the synthesis of ribonucleic acid catalyzed by ribonucleic acid polymerase in *Azotobacter vinelandii* (KRAKOW 1963). This stimulation however depended on the nature of the primer and took place only when the reaction was primed by native deoxyribonucleic acid. With synthetic polyribonucleotides as the primer there was even an inhibition of the reaction indicating that the mechanism for this stimulation was not due to inhibition of the nucleases contaminating the polymerase preparation. Similar stimulation of ribonucleic acid synthesis by polyamines was also observed in *Micrococcus lysodeikticus* (FOX and WEISS 1964). Spermine has a stimulatory effect on deoxyribonucleic acid polymerase reaction in *Physarum polycephalum* (BREWER and RUSCH 1966).

The patterns of cellular spermidine and ribonucleic acid in a polyauxotrophic mutant of *E. coli* changed in a similar manner under various nutritional conditions and the addition of spermidine to a medium devoid of the amino acid arginine caused a several fold stimulation in the ribonucleic acid synthesis. The elimination of arginine from the medium not only inhibited ribonucleic acid synthesis but also prevented the cellular accumulation of spermidine. Putrescine under these conditions was only slightly stimulatory and the stimulatory effect of spermidine on ribonucleic acid synthesis was gradually abolished by increasing the concentration of putrescine in the medium (RAINA and COHEN 1966).

Using an isogenic pair of relaxed and stringent auxotrophic strains of

*E. coli*: COHEN HOFFNER JANSEN MOORE and RAINA (1967) could further show a close relationship between spermidine and ribonucleic acid synthesis. In amino acid starved cultures the relaxed strain produced greater quantities of both ribonucleic acid and spermidine compared to the stringent strain. The synthesis of spermidine in the relaxed strain was independent of ribonucleic acid accumulation.

The action of putrescine and polyamines on ribosomes and protein synthesis is covered by a relatively well documented area in the literature concerning their physiological function. Many correlations between the effects of putrescine and polyamines and divalent inorganic cations at the cellular and subcellular level have been observed. As stated previously the ribosomes of both bacterial and animal cells contain considerable amounts of putrescine, spermidine and spermine (COHEN and LICHTENSTEIN 1960, ZILLIG, KRONE and ALBERS 1959, RAINA and TELARANTA 1967). These compounds have been observed to cause similar ribosomal aggregations and stimulation of protein synthesis as do the divalent cations (HERSCHKO, AMOZ and MAGER 1961, NOLLER and LIM 1965). On the other hand the completely opposite effect, an inhibition of protein synthesis, has also been reported. Spermine inhibited protein synthesis in Walker 256 carcinosarcoma under the same culture conditions as it stimulated ribonucleic acid synthesis (GOLDSTEIN 1965). Spermine also inhibits polypeptide synthesis in a subcellular system derived from the L 1210 mouse ascites leukemia (OCHOA and WEINSTEIN 1965) and protein synthesis in growing cells of *Staphylococcus aureus* (FRIEDMAN and BACHRACH 1966). The effects of polyamines on protein synthesis thus seems to be critically dependent on their concentration: low concentrations stimulate and high concentrations inhibit protein synthesis.

Oxidized spermine is toxic to various bacteria, inactivates bacterial and plant viruses (TABOR and TABOR 1964) and inhibits the multiplication of Ehrlich ascites cells (BACHRACH, ABZUG and BEKIERKUNST 1967).

*Relationships between polyamines and nucleic acids in animal tissues*. Most of the observations indicating connections between polyamines and nucleic acids have been obtained from microbiological material, i.e. from bacteria and viruses. On the other hand there are only a few, and in most cases indirect studies concerning this relationship in animal tissues. The assumptions suggesting an association between polyamines and nucleic acids, especially with ribonucleic acid in animal tissues, are almost without exception based on parallel changes found in polyamine and nucleic acid concentrations under certain experimental conditions.

The concentration of polyamines showed similar changes to those shown by nucleic acids during the development of the chick embryo (RAINA 1963, CALDARERA, BARBIROLI and MORUZZI 1965). In addition to the polyamines the diamines putrescine and cadaverine also showed increases and decreases preceding those observed in nucleic acids at various developmental stages (CALDARERA, BARBIROLI and MORUZZI 1965). This parallel behaviour between polyamines and nucleic acids was not entirely limited to physiological developmental conditions in the embryo. Some unphysiological experimental

conditions which varied the polyamine pattern also caused similar changes in nucleic acids

As pointed out in previous sections spermidine concentration was at its highest in rat tissues at birth thereafter gradually decreasing with age (JÄNNE RAINA and SIIMES 1964) This uniform feature observed in each tissue studied shows some similarities to the changes in nucleic acids especially ribonucleic acid taking place during the postnatal development of the rat (FUJII and KOYAMA 1962 OLIVER BALLARD SHIELD and BENTLEY 1962)

If the polyamines in animal tissues too are in some way related to nucleic acids such a connection is not so obvious during the normal developmental growth of the animal On the other hand this correlation is much more apparent in tissues subjected to extreme experimental conditions e.g. in rat liver undergoing regenerative growth

When the medial and left lateral lobes of the rat liver have been removed the remaining lobes undergo a process of rapid growth resulting in an almost complete regeneration of the original tissue mass within a few weeks During this period of regeneration many biochemically interesting alterations take place rapidly The first changes occur in the areas of glycogen and lipid metabolism (HARVESS 1957) and in the biosynthesis of ribonucleic acid Partial hepatectomy of the rat also provokes rapid changes in the field of polyamine metabolism (DYKSTRA and HERBST 1965 RAINA JÄNNE and SIIMES 1965 1966) During the regeneration process spermidine concentration like that of ribonucleic acid increased very sharply reaching a maximum at 64 h after partial hepatectomy There was also early stimulation in the biosynthesis of this polyamine which was indicated by a marked rise in the incorporation of radioactive precursors into spermidine (DYKSTRA and HERBST 1965 RAINA JÄNNE and SIIMES 1965 1966) A clear increase in synthesis occurred as early as 8 h after partial hepatectomy and a net increase in liver spermidine could be seen at 16 h The behaviour of ribonucleic acid content in regenerating liver closely resembled that of spermidine On the other hand the concentration of spermine (per wet wt unit) like that of deoxyribonucleic acid decreased significantly during early regeneration and an increase in their concentration exceeding the control values could not be seen until 9 days after partial hepatectomy (RAINA JÄNNE and SIIMES 1966)

Thus there is an obvious correlation between polyamines and ribonucleic acid in animal tissues too but the exact function of these compounds is almost completely obscure However on the knowledge we have of them in the microbiological world we can assume that polyamines also exert similar stabilizing and neutralizing effects on nucleic acids in animal tissues as they do in bacteria and viruses This hypothesis is strongly supported by the discovery that during the whole period of regeneration the ratio of polyamine nitrogen to nucleic acid phosphate especially ribonucleic acid remained quite constant (RAINA JÄNNE and SIIMES 1966)

There are in addition to regenerating liver some other conditions under which the behaviour of polyamines and nucleic acids closely resemble each other CALDARERA COZZANI and MORUZZI (1966) showed recently that in rat livers subjected to ionizing radiation there was a considerable increase in the



content of both polyamines a lesser increase in ribonucleic acid while deoxyribonucleic acid showed less evident changes

An injection of growth hormone into rats provokes many anabolic effects in the liver tissue including a marked stimulation of ribonucleic acid synthesis (TALWAR PANDA SARIN and TOLANI 1962 KORNER 1963) The content of liver polyamines are also affected by this hormone (KOSTYO 1965) The changes in hepatic polyamine concentrations followed by growth hormone treatment were essentially similar to those observed in regenerating liver spermidine increased but spermine decreased or remained unchanged On the other hand after hypophysectomy the situation was just the reverse spermidine decreased and spermine increased slightly

In this connection the close subcellular distribution of polyamines and ribonucleic acid in rat liver should also be mentioned (RAINA and TELA RANTA 1967)

An interesting effect of these compounds is their ability to serve as growth factors for a mammalian cell line also indicating their importance even for animal tissues (HAM 1964)

On the results of the several investigations cited in this section it could be concluded that there are undoubtable relationships between putrescine spermidine spermine and nucleic acids in microorganisms as well as in animal tissues The simplest explanation of some of these phenomena is that based on the ionic affinity between positively charged polyamines and negatively charged nucleic acids thus resulting in a neutralization between these compounds On the other hand there are also many reports indicating even a primary regulation of nucleic acid metabolism by polyamines

## Outline of present study

As stated in previous sections, polyamines and diamines are widely distributed not only in microorganisms but also in animal tissues. The knowledge we have of their possible physiological role and significance is almost entirely based on studies carried out on microorganisms.

A great deal of attention has also been paid to the occurrence, biosynthesis and behaviour of these compounds in the microbiological world. In recent years, however, increasing evidence has indicated their importance in animal tissues too. On the other hand, relatively little is known of their biosynthesis in animal tissues or the association of their biosynthetic pathways with the intermediary metabolism of animals.

The polyamines spermidine and spermine have been shown to be synthesized in animal tissues from the same precursors as in microorganisms. The amino acids ornithine, arginine and methionine belong to the normal constituents of an animal organism. Although there are reports of the isolation of putrescine from mammalian tissues, it is questionable whether this diamine occurs in sufficient amounts to serve as a natural precursor of polyamines in mammalian organisms too.

The behaviour of polyamines in a rapidly growing animal tissue is an interesting feature in their metabolism. What is the sequence of the events culminating in a marked increase in the concentration of spermidine in regenerating liver or in hepatic tissue after growth hormone treatment?

The present investigation is divided into three parts:

- I The occurrence of the diamine putrescine in normal rat liver at different ages and its conversion *in vivo* and *in vitro* into hepatic polyamines in normal and ethionine-treated rats
- II The changes taking place in putrescine and polyamine contents during early liver regeneration and the alterations in various biosynthetic steps involving polyamine biosynthesis
- III The effect of pituitary growth hormone on putrescine and polyamine contents as well as on their biosynthesis in rat liver

# Material and methods

## EXPERIMENTAL ANIMALS

The animals used were albino rats of Wistar strain (an inbred strain from this laboratory) or as in the hormone experiments of Sprague Dawley strain (obtained from Oy Orion Ab Mankkaa Finland) The rats were kept on a routine laboratory diet (Hankkija Oy Helsinki Finland) In general the animals were allowed to eat and drink ad libitum except those used in the hormone experiments which fasted 24 hours before killing The age which seems to be an important factor affecting the metabolism of polyamines varied in different experiments and will be mentioned separately with each experiment The rats were killed by decapitation before assay procedures

## IN VIVO TECHNIQUES

*Partial hepatectomy* Partial hepatectomy of the rat was performed essentially as described by HIGGINS and ANDERSON (1931) Under light ether anaesthesia the medial and left lateral lobes were removed The removed part of the liver was approximately equal to two thirds of the whole rat liver The mortality rate during or after this relatively simple surgical procedure was minimal No special post operative care was found to be necessary and the animals were allowed to eat and drink ad libitum immediately after operation

In preliminary experiments it was observed that the laparotomy or anaesthesia alone had no effect on putrescine and polyamine biosynthesis Thus the control groups of various experiments were represented by unoperated rats From those control animals only the right lateral and caudate lobes (the remaining part after partial hepatectomy) were used for analysis In each experiment the rats were decapitated at the same time the precursors were injected at the same time but the operations were performed 2 to 72 hours before analysis

*Treatment with growth hormone* The pituitary growth hormone used was a commercial preparation (Somacton Ferring Malmo Sweden) This drug had a relatively high degree of purity containing approximately 1 international unit per 0.5 mg of dry substance (personal communication by Ferring) The hormone was dissolved in a small volume of its original solvent containing 0.250 g glucose 0.025 g phenol and HCl ad pH 4 in 5 ml of distilled water and there after diluted 4 fold with 0.9 per cent NaCl The drug was administered intra

peritoneally in a volume of 2 ml. The control animals received the solvent only in equal volume and dilution. In all experiments the animals were fasted for 24 hours before decapitation.

## IN VITRO TECHNIQUES

After decapitation of the rats the livers used for *in vitro* experiments were immediately perfused *in situ* with cold physiological saline and placed in ice-cold buffer at 3°C.

In a few preliminary experiments tissue slices of rat liver were used. The slices were prepared at 3°C with a slicer described by STADIE and RIGGS (1944). The tissue pieces were then weighed and transferred into flasks containing ice-cold Krebs Ringer phosphate buffer ( $\text{Ca}^{++}$  omitted) at pH 7.4. 300 or 500 mg of slices were used for each incubation. After incubation the slices were washed twice with cold buffer, homogenized in 0.1 N HCl and analyzed for the radioactivity in polyamine fractions.

In the majority of the preliminary experiments and in all the experiments presented here liver homogenates were used. After perfusion and excision the livers were immediately homogenized with a Potter Elvehjem type homogenizer. The homogenization medium was usually 250 mM sucrose containing 10 mM potassium phosphate buffer at an initial pH of 7.8 and the homogenates contained 40 to 50 per cent (wt/vol) liver tissue. The incubations were performed in 25 ml flasks usually in a volume of 2 ml in an incubator with mechanical shaking. The reaction was stopped by adding 4 ml of 0.1 N HCl to the flasks and placing them in ice at 3°C.

The blanks were kept in ice at 3°C during the whole period of incubation.

To exclude possible bacterial contamination in the incubations a series of liver homogenates was prepared and incubated under sterile conditions. These sterile preparations showed essentially similar spermidine synthesizing capacity as did those prepared in the normal manner.

## ANALYTICAL METHODS

**Reagents.** The reagents used were of analytical grade in most instances obtained from Merck AG (Darmstadt, Germany).

**Reference standards.** Putrescine dihydrochloride, spermidine phosphate and spermine tetrahydrochloride were obtained from the Mann Research Laboratories (New York, U.S.A.). The albumin standard for protein measurements was obtained from Kabi (Stockholm, Sweden).

**Isotopes.** All the isotopes used were purchased from the New England Nuclear Corporation (Boston, Massachusetts, U.S.A.). The following radioactive precursors of putrescine and polyamines were used: DL arginine-5  $^{14}\text{C}$  (in 0.1 N HCl) specific activity 50 mC/mmole; DL methionine-2  $^{14}\text{C}$  specific activity 481 mC/mmole; DL ornithine-5  $^{14}\text{C}$  (in 0.1 N HCl) specific activity 98 mC/mmole and putrescine 14  $^{14}\text{C}$  (as dihydrochloride) specific activity 950 or 5...

mC/muole. The radioactive stock solutions were stored at  $-20^{\circ}\text{C}$  and diluted before use with 0.9 per cent NaCl or with appropriate buffer in *in vitro* experiments. The isotopes were administered intraperitoneally in each *in vivo* experiment.

*Determination of putrescine and spermine from tissue samples* The polyamines were determined by the method introduced by RAINA (1963) and later modified by JÄNNE, RAINA and SIIMES (1964). The procedure is briefly as follows. After homogenization and deproteinization of a tissue sample the polyamines were extracted from a strongly alkaline solution into  $\text{m}$  butanol. The butanol phase was then evaporated to dryness, the residue dissolved in a small volume of 0.1 N HCl and stored in a small test tube until use. The final separation of the amines was performed by paper electrophoresis using 0.1 M citric acid buffer at pH 3.6 or 4.3. The mean recoveries in the butanol extraction were 92 and 95 per cent respectively for spermidine and spermine. The appropriate corrections for polyamines due to loss during extraction were applied.

The staining of polyamines with amido black washing procedures and elution of the stained fractions have been previously described (JÄNNE, RAINA and SIIMES 1964).

*Determination of putrescine* Putrescine was determined from the same samples as polyamines but always using 0.1 M citric acid buffer at pH 4.3 in the paper electrophoresis. The paper strips were stained with ninhydrin in acetone (RAINA and COHEN 1966) instead of amido black. The coloured fractions were then eluted with 6 ml of water-ethanol-glacial acetic acid 1:4:5 (by vol) containing 2 mg of cadmium acetate per ml. The extracted red colour which showed maximum absorption at 505 m $\mu$  was found to be completely stable when kept in dark for at least three hours. The colour was measured within one hour with a Beckman II spectrophotometer at 505 m $\mu$ .

The mean recovery of putrescine in the butanol extraction was 86 per cent and the appropriate correction was applied.

*Protein measurements* Proteins were determined from the washed trichloroacetic acid precipitate according to LOWRY, ROSEBROUGH, FARR and RANDALL (1951) using human albumin as a reference standard.

*Radioactivity measurements* Putrescine and polyamines were counted directly from the electropherograms after staining the strips with 0.1 per cent ninhydrin in ethanol in a Packard Tri Carb liquid scintillation spectrometer (Packard Instrument Company Inc., La Grange, Illinois, U.S.A.). As a liquid scintillator 2 ml of 0.3 per cent PPO ( $\sim$ 5 diphenyloxazole) and 0.01 per cent dimethyl POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene] (Packard Instrument Company) in toluene was used.

The radioactivity of liver protein was also counted from paper strips. The washed trichloroacetic acid precipitate was first dissolved with 0.1 N NaOH. Thereafter a sample of 0.1 ml was applied onto a small band of filter paper (Whatman No. 1) and counted using the above liquid scintillator with an efficiency essentially equal to that with putrescine and polyamines (about 55 per cent).

*Paper chromatography of putrescine and polyamines* The identity and purity of the amine fractions obtained from routine electrophoresis was checked

as follows. The amines were first separated, purified and somewhat concentrated by a preparative paper electrophoresis. 0.3 to 0.5 ml of an unknown sample was applied onto a 15×39 cm paper strip and run in electrophoresis in the normal manner. After drying the papers, the fractions were visualized by drawing a few ninhydrin lines with a micropipette along the migration direction of the amines. Thereafter, the papers were dried at 105°C and the amine fractions were cut off according to the stained ninhydrin lines. The unstained parts of the fractions were then eluted with 10 ml of 0.1 N HCl; the eluate was made strongly alkaline and the amines were then extracted into n-butanol. The butanol phase was evaporated to dryness and the rest was dissolved in a small volume of 0.1 N HCl. The purified fractions were identified by paper chromatography using one-dimensional ascending or descending techniques. The following solvent systems were used:

- I n-butanol:acetic acid:pyridine:water (4:1:1:2)
- II n-propanol:conc. HCl:water (3:1:1)
- III n-butanol:acetic acid:water (2:1:1)

In solvent I and II, the ascending technique was used. The running time was 8 to 10 hours. With solvent III, the descending technique and a running time of 36 hours was used. The stained spots were then compared with the authentic standards which had undergone a similar purification procedure. Only one ninhydrin-positive spot could be detected in the chromatograms obtained from a purified electrophoretic fraction. The  $R_f$  values of the spots corresponded to those of the standards in each studied chromatographic system.

The radiopurity of putrescine, spermidine and spermine was tested according to the above purification procedure. The specific activities of putrescine or polyamines from an unknown sample, obtained by a single routine paper electrophoresis, were essentially the same after the purification procedure.

To exclude the presence of acetylated derivatives of putrescine and polyamines (DUBIN and ROSENTHAL 1960), various samples were subjected to acid hydrolysis. To each butanol residue, originally dissolved in 0.1 N HCl, an equal volume of conc. HCl was added; the test tubes were sealed and placed in an oven for 12 hours at 105°C. Thereafter, the samples were evaporated to dryness, dissolved in the original volume of 0.1 N HCl and analyzed for their putrescine and polyamine contents. No evidence of the presence of acetylated derivatives of putrescine or polyamines was obtained.

*Statistical procedures.* The presented values are arithmetic means, usually with the ranges or  $\pm$  standard deviations. The significance of the differences between the groups are estimated by Student's *t* test.

# Results

## I OCCURRENCE OF PUTRESCINE AND ITS CONVERSION INTO POLYAMINES IN RAT LIVER

Putrescine, the common diamine in microorganisms can act as a precursor in polyamine biosynthesis also in animal tissues (RAINA 1963, 1964, DYKSTRA and HERBST 1965, JÄNNE and RAINA 1966). This diamine has also been reported as occurring in considerable amounts in developing chick embryos (CALDARERA, BARBIROLI and MORUZZI 1965). Although some previous studies have dealt with the occurrence of putrescine in mammalian tissues (WEAVER and HERBST 1958, FISCHER and BOHN 1957) the knowledge we have of the distribution of this compound in mammalian tissues is rather limited. Earlier observations indicated that the concentration of putrescine in the pancreas and liver of some mammalian species was very small compared with that of spermidine and spermine. These observations question the importance of putrescine in mammalian tissues as a natural precursor in polyamine synthesis or suggest a rapid turnover rate of this compound.

The next experiments deal with the occurrence of putrescine and its conversion into polyamines in rat liver under some physiological and experimental conditions.

### *Concentration of putrescine in liver at different ages*

Putrescine was found to occur in all the liver samples analyzed even if at a rather low concentration. As shown in Fig. 2 its concentration varied markedly with age. The hepatic concentration of putrescine which during the first two weeks of life was under 100  $\mu$ moles per g wet wt increased sharply thereafter and reached a maximum of 70  $\mu$ moles/g between the first and second month. Further increase in age again clearly decreased liver putrescine which in 9 month old rats had diminished to the level found in young rats.

It is obvious that in general changes in the concentration of liver putrescine with increasing age do not follow those found in liver polyamines (not tabulated). For example in the above material the concentration of spermidine was at its highest immediately after birth i.e. 1170  $\mu$ moles/g wet wt and then decreased rapidly being only 460  $\mu$ moles at the age

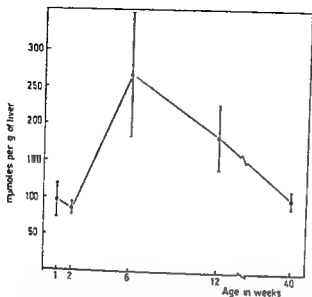


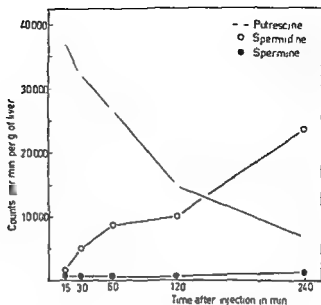
Fig. 2 The concentration of liver putrescine in relation to age. Each age point represents a mean obtained from five to six male rats except that of the 40 week group which contained five pooled samples from four organs in each. The vertical lines represent standard deviations of the means.

At 40 weeks the concentration of putrescine was thus relatively low compared with that of spermidine varying from about one tenth to one third at various stages of development.

#### Conversion of putrescine into polyamines in rat liver *in vivo*

The biosynthesis of polyamines from exogenously added putrescine in normal rat liver is demonstrated in Fig. 3. The animals used in this experiment were two weeks old and received  $^{14}\text{C}$  putrescine as an intraperitoneal injection 15 to 240 min before analysis. As shown in the figure the radioactivity of liver putrescine decreased relatively rapidly while that of polyamines increased continuously. Between 15 and 240 min the activity of putrescine expressed as counts/min per g wet wt decreased from 37100 to 6700 and that of spermidine increased from 1600 to 1800 respectively. The radioactivity of liver spermine however barely exceeded that of the background even at 4 h after the putrescine injection. Thus between 15 and 240 min the activity of liver putrescine decreased by 2400 counts/min per g and that counted in polyamine fractions increased by 22200 counts/min per g, i.e. at 4 h after the putrescine injection the





*Fig. 3 The conversion of putrescine into polyamines in rat liver. Two week old male rats received 2  $\mu$ C (= 0.21  $\mu$ mole) of putrescine-1,4  $^{14}$ C as an intra peritoneal injection. Each value represents a mean of three animals.*

activity of the hepatic polyamines represented about 73 per cent of that having disappeared from the liver putrescine between 15 min and 4 h. On the other hand the radioactivity of the total liver polyamines represented only a small portion (2–3 per cent) of the  $^{14}$ C putrescine dose. Accordingly it seems that there are other routes for the metabolism of exogenously added putrescine in mammalian organism.

Another fact also shown in Fig. 3 was the relatively rapid disappearance of putrescine from hepatic tissue suggesting a rapid turnover rate of this compound. This in turn could partly explain the low concentration of putrescine as compared with those of spermidine and spermine whose turnover rates are much slower (JÄNNE and RAINA 1966, SIIMES 1966, 1967).

#### *Fate of the radioactivity from exogenously administered $^{14}$ C putrescine*

As pointed out above the radioactivity of the polyamines synthesized from  $^{14}$ C putrescine in rat liver represented only a small percentage of that of the injected isotope. It seems thus that there are other routes for the elimination of exogenous putrescine in mammalian tissues.

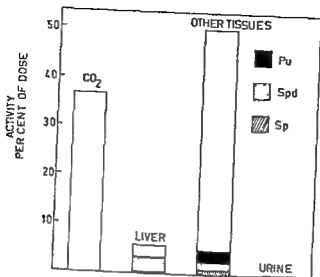


Fig 4 The fate of radioactivity derived from exogenously administered  $^{14}\text{C}$  putrescine

Three two month old male rats received  $5\ \mu\text{C}$  ( $= 0.53\ \mu\text{mole}$ ) putrescine  $^{14}\text{C}$  as an intraperitoneal injection 2 h before decapitation. Expired  $^{14}\text{CO}$  was collected into 4 N NaOH and counted from paper strips. The total radioactivity of the liver and other tissues ( $=$  rat tissue without liver and skin) represents the activity counted in trichloroacetic acid soluble and insoluble fractions. The activities were counted from paper strips.  $^{14}\text{C}$  putrescine was used as internal standard. Pu = putrescine Spd = spermidine Sp = spermine.

Fig 4 presents the total distribution of the radioactivity at 2 h after injection with  $^{14}\text{C}$  putrescine. As shown in the figure, over one third of the injected dose was expired as  $^{14}\text{CO}$  within 2 hours. Only about 6 per cent of the total dose was retained in the liver at 2 h after injection. On the other hand, more than one half of the activity recovered from the liver was found in putrescine and polyamines, mainly in the spermidine fraction. Most of the radioactivity, however, was found in the remaining tissues (other tissues except liver and skin), but in comparison with the liver, the radioactivity of putrescine and polyamines represented only approximately one tenth of the total radioactivity recovered from those tissues.

A very rapid oxidation thus seems to be the major elimination route for exogenously administered putrescine. The location of this oxidation is not known, nor whether it occurs extracellularly or intracellularly.

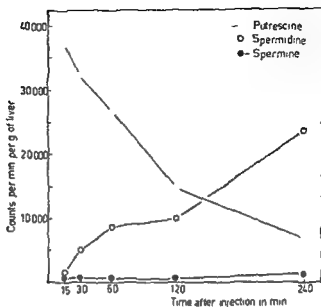


Fig 3 The conversion of putrescine into polyamines in rat liver Two-week-old male rats received  $\mu\text{C}$  ( $= 0.21 \mu\text{mole}$ ) of putrescine  $2-4^{14}\text{C}$  as an intraperitoneal injection Each value represents a mean of three animals

activity of the hepatic polyamines represented about 73 per cent of that having disappeared from the liver putrescine between 15 min and 4 h On the other hand the radioactivity of the total liver polyamines represented only a small portion (2–3 per cent) of the  $^{14}\text{C}$  putrescine dose Accordingly it seems that there are other routes for the metabolism of exogenously added putrescine in mammalian organism

An other fact also shown in Fig 3 was the relatively rapid disappearance of putrescine from hepatic tissue suggesting a rapid turnover rate of this compound Thus in turn could partly explain the low concentration of putrescine as compared with those of spermidine and spermine whose turnover rates are much slower (JÄNNE and RAINA 1966 SIIMES 1966 1967)

#### *Fate of the radioactivity from exogenously administered $^{14}\text{C}$ putrescine*

As pointed out above the radioactivity of the polyamines synthesized from  $^{14}\text{C}$  putrescine in rat liver represented only a small percentage of that of the injected isotope It seems thus that there are other routes for the elimination of exogenous putrescine in mammalian tissues

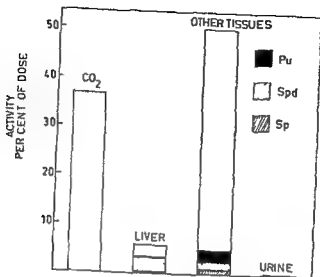


Fig 4 The fate of radioactivity derived from exogenously administered  $^{14}\text{C}$  putrescine

Three two month old male rats received  $5\ \mu\text{C}$  ( $= 0.53\ \mu\text{mole}$ ) putrescine  $^{14}\text{C}$  as an intraperitoneal injection 2 h before decapitation. Expired  $^{14}\text{CO}$  was collected into 4 N NaOH and counted from paper strips. The total radioactivity of the liver and other tissues (= rat tissues without liver and skin) represents the activity counted in trichloroacetic acid soluble and insoluble fractions. The activities were counted from paper strips.  $^{14}\text{C}$  putrescine was used as internal standard. Pu = putrescine, Spd = spermidine, Sp = spermin.

Fig 4 presents the total distribution of the radioactivity at 2 h after injection with  $^{14}\text{C}$  putrescine. As shown in the figure, over one third of the injected dose was expired as  $^{14}\text{CO}$  within 2 hours. Only about 6 per cent of the total dose was retained in the liver at 2 h after injection. On the other hand, more than one half of the activity recovered from the liver was found in putrescine and polyamines, mainly in spermidine fraction. Most of the radioactivity, however, was found in the remaining tissues (other tissues except liver and skin), but in comparison with the liver, the radioactivity of putrescine and polyamines represented only approximately one tenth of the total radioactivity recovered from those tissues.

A very rapid oxidation thus seems to be the major elimination route for exogenously administered putrescine. The location of this oxidation is not known, nor whether it occurs extracellularly or intracellularly.

Table 1 lists the activities of the liver and other tissues expressed as counts/min per g of tissue wet wt. As shown, the radioactivity in the liver tissue was about twice that in the other tissues. In the liver the radioactivity found in spermidine comprised about 40 per cent of the total activity, whereas in the other tissues it was only 3 per cent. The specific activity of spermidine isolated from the liver was 31 100 count./min per  $\mu$ mole and that from the other tissues 6 900 respectively. The activities without putrescine and polyamines were approximately equal in both hepatic and other tissues, i.e. 38 800 counts/min per g wet wt in the liver and 36 200 in the other tissues.

This experiment does not exclude the liver as the place for the oxidation of putrescine, but it does somewhat suggest that an important pathway in the hepatic metabolism of putrescine is its conversion into liver polyamines.

*Table 1. The fate of radioactivity derived from exogenously administered  $^{14}$ C putrescine. See legend for Fig. 4.*

	Activity Counts/min per g wet wt	
	Liver	Other tissues
Total	81 500	40 200
Putrescine	4 800	1 900
Spermidine	34 600	1 300
Spermine	3 300	800

#### *Biosynthesis of spermidine from putrescine in liver homogenates*

In the preliminary experiments it was observed that tissue slices of the rat liver or liver homogenate could synthesize spermidine from putrescine or methionine. The rate of the biosynthesis of spermidine from putrescine in liver slices was however much slower than that in the intact tissue and also slower than in liver homogenates. In general, the incorporation of putrescine into spermidine *in vitro* proceeded without any special additions. Neither was the incorporation stimulated

by the addition of glucose adenosine triphosphate or methionine. Minor changes in ionic strength did not alter the synthesizing capacity either. Two different incubation media were generally used for liver homogenates: a) An ionic medium containing 116 mM  $K^+$ , 8 mM  $Na^+$ , 16 mM  $Mg^{++}$ , 124 mM  $Cl^-$ , 16 mM  $SO_4^{--}$  and buffered with 10 mM potassium phosphate buffer at an initial pH of 7.8, thus resembling the physiological intracellular medium; b) A crude sucrose medium containing 250 mM sucrose and buffered with 10 mM potassium phosphate buffer at an initial pH of 7.8. Both these media in which the formation of spermidine from putrescine was approximately equal were used in the present study.

Fig. 5 presents the effect of the incubation time on the incorporation of the label from  $^{14}C$ -putrescine into spermidine in liver homogenates obtained from two-month old rats. Accordingly, labelled spermidine could soon be isolated from the homogenates incubated in a medium containing radioactive putrescine. The radioactivity of spermidine seemed to increase

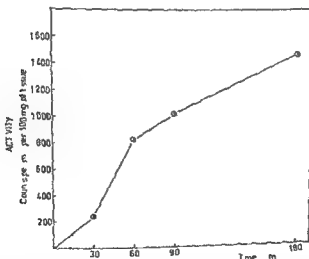


Fig. 5 The synthesis of spermidine from putrescine in liver homogenates. The homogenate was pooled from three male rats aged two months. Each 25 ml flask contained 1.0 g of liver tissue (wet wt) and 0.0168 mM ( $\approx 0.16 \mu C/ml$ ) putrescine-1,4- $^{14}C$  in 250 mM sucrose buffered with 10 mM potassium phosphate buffer at pH 7.8 in a final volume of 2.5 ml. Incubation was performed at 37°C using as gas phase atmospheric air and using mechanical shaking. The reaction was stopped by adding 4 ml of 0.1 N HCl to the flasks and placing them in ice. Each time point represents a mean of three assays.

for at least three hours. No radioactivity could be detected in spermine fraction under these experimental conditions. The rate of conversion of putrescine into spermidine apparently seemed to be slower in the liver homogenate *in vitro* than *in vivo* although the experimental conditions are not directly comparable because of different substrate concentrations. According to Fig. 5 the total radioactivity per flask found in the spermidine fraction after a 3 hour incubation period represented only about 2-3 per cent of that of the added putrescine. The radioactivity found in CO under the same experimental conditions was negligible.

As stated in previous sections (p 10 and Fig. 4) the age of an animal seems to be an important factor affecting putrescine and polyamine metabolism. Fig. 6 illustrates the biosynthesis of spermidine from putrescine in liver homogenates in relation to age. The conversion of putrescine into spermidine was relatively slow during the first weeks after birth but then increased rapidly up to the age of 10 weeks. On the other hand there was a sudden decrease in spermidine synthesis between 10 and 14 weeks and this slower rate of synthesis seemed to remain until adulthood. The changes occurring during the process of aging were quite considerable since more than two fold increase in spermidine biosynthesis from putrescine could be observed between 2 and 10 weeks. Thus the changes in spermidine synthesis as measured by the incorporation of putrescine into spermidine in liver homogenates showed some resemblance to the changes in the concentration of putrescine during postnatal development (Fig. 2). If this is also valid in an intact organism the accumulation of putrescine in rat liver between 2 and 6 weeks can not be explained in terms of a decrease in the conversion of putrescine into spermidine, and it must be assumed that the biosynthesis of putrescine from its precursors is markedly increased at that time or that the other metabolic routes of putrescine are changed.

The above pattern of spermidine biosynthesis from putrescine does not correspond with the concentration curve of spermidine as shown in Fig. 7A. In fact the concentration of spermidine decreased continuously with age despite the marked increase in its synthesis from putrescine *in vitro* between 2 and 10 weeks. The discrepancy between the concentration and biosynthesis of spermidine during early postnatal development could be explained by assuming that the formation of spermine from spermidine is markedly increased during the first weeks after birth. This idea is supported by the finding that the concentration of spermine increased from 260  $\mu$ moles/g wet wt to 650 between 2 and 5 weeks i.e. more than two fold.

while that of spermidine decreased only from 700  $\mu$ moles/g to 620 at the same time (Fig 6 A)

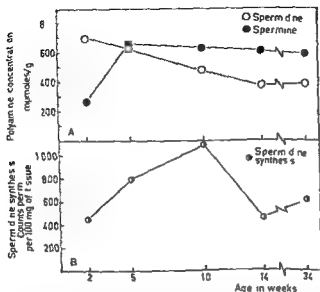


Fig 6 A The hepatic concentrations of spermidine and spermine in relation to age Four pooled samples obtained from three male rats in each group except the 2 week's age group which contained four pooled samples from eight rats  
Fig 6 B Spermidine synthesis from putrescine in liver homogenates in relation to age The same material as in Fig 6 A The assay conditions as in Fig 5 except using 0.8 g tissue and 0.0165 ml ( $\approx 0.15 \mu$ C/ml) putrescine  $2,4-^{14}$ C in a final volume of 2 ml The incubation time was 60 min Each age point represent a mean of four assays

### The early effect of ethionine on the biosynthesis of spermidine

There are some indirect possibilities for the study of the biosynthetic sequences of polyamine metabolism As stated previously methionine can act as a precursor of polyamine synthesis even in animal tissues This amino acid is first converted into S adenosylmethionine at least in the microorganisms and thereafter decarboxylated to form the propylamine moiety for spermidine or spermine molecule Ethionine an ethyl analogue of methionine causes a series of metabolic alterations in animal tissue (STEKOL 1963) Many of these effect can be attributed to the formation of S adenosylethionine (MODY BULBA HOLOWECKY and STEKOL 1963 RAINA JÄNNE and SIIMES 1964) and the marked decrease in tissue adenosine triphosphate level (SHULL 1962 BARTELS and HOHORST



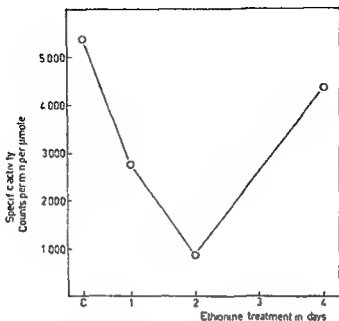


Fig 6 The effect of ethionine on spermidine synthesis from putrescine in rat liver *in vivo*. The animals were one month old male rats receiving daily 0.2 mmole of DL ethionine as an intraperitoneal injection. Analysis 3 h after injection with 1.5  $\mu\text{C}$  ( $= 0.10 \mu\text{mole}$ ) of putrescine 1-4  $^{14}\text{C}$ . Three animals in each group.

1963). Ethionine also causes considerable changes in liver polyamines in chick embryos (RAINA 1963) as well as in the rat (RAINA, JÄNNE and SIIMES 1964). Ethionine treatment first caused a decrease in liver spermidine and spermine followed by a continued decrease in spermine and marked increase in spermidine concentration. Considering that methionine serves as the precursor for polyamines, this early decrease in spermidine concentration could be explained by the formation of S-adenosylethionine and a decrease in the level of S-adenosylmethionine in the tissues. The later accumulation of spermidine and simultaneous decrease in spermine concentration might be due to a persistent block between spermidine and spermine (RAINA, JÄNNE and SIIMES 1964; SIIMES 1967) at the same time as the synthesis of spermidine is resumed.

Fig 7 illustrates the marked effect of ethionine treatment on the biosynthesis of spermidine from putrescine in rat liver *in vivo*. After two days' treatment with an ethionine dose of 0.2 mmole/animal per day, a drastic decrease in the incorporation of radioactivity from  $^{14}\text{C}$  putrescine

into spermidine was obvious. The specific activity of liver spermidine decreased from the control value 5400 counts/min per  $\mu$ mole to 770 at 2 days i.e. by almost 90 per cent and thereafter again increased towards the control value at 4 days after the beginning with the ethionine treatment. In this context it should be mentioned that no additional increase in incorporation exceeding the control value could be observed by employing a longer observation period or a larger dose of ethionine. It seems thus that the inhibition of spermidine synthesis after ethionine treatment was only transient and the synthesis was completely resumed within a few days. This behaviour in spermidine synthesis after ethionine treatment suggests that S-adenosylmethionine acts with putrescine as the precursors of spermidine in animal tissues too and that the reduction of this synthesis during the first days of ethionine treatment is due to the formation of S-adenosylethionine which probably not can serve as a three-carbon moiety for spermidine synthesis. On the other hand the ability of

Table 1. Effect of methionine and adenosine triphosphate (ATP) on the incorporation of label from  $^{14}$ C putrescine into spermidine in liver homogenates obtained from saline and ethionine treated rats

The animals were three month old female rats. Two of them received an intraperitoneal injection of physiological saline and two 0.625 mmole DL ethionine 3 h before incubation. Each 25 ml flask contained 10 g of liver tissue (wet wt): 116 mM  $K^+$ , 16 mM  $Mg^{++}$ , 8 mM  $Na^+$ , 124 mM  $Cl^-$ , 16 mM  $SO_4^{--}$ , 10 mM potassium phosphate buffer at pH 7.8, 0.011 mM ( $=0.1 \mu$ C/ml) putrescine  $^{14}$ C and additions as indicated in a final volume of 2 ml. Incubation time was 60 min. Other conditions as in Fig. 5. The values are means  $\pm$  standard deviations (SD) of three assays.

Pretreatment	Additions	Spermidine activity Counts/min per 100 mg of tissue	Change %
Saline	—	490 $\pm$ 94	$\pm$ 0
Saline	DL methionine 0.2 $\mu$ mole	445 $\pm$ 23	- 9
Saline	ATP 2.0 $\mu$ moles	410 $\pm$ 80	-16
Ethionine	—	170 $\pm$ 38	$\pm$ 0
Ethionine	DL methionine 0.2 $\mu$ mole	215 $\pm$ 12	+26
Ethionine	ATP 2.0 $\mu$ moles	230 $\pm$ 4	+35

S-adenosylethionine to be incorporated into the spermidine molecule at least after longer treatment is not excluded

Table 2 attempts to show directly a possible lack of available methionine and adenosine triphosphate in the livers of rats treated with ethionine. As shown in the table pretreatment with ethionine also caused a marked decrease in the capacity of liver homogenates to synthesize spermidine from  $^{14}\text{C}$  putrescine. As early as 3 hours after a relatively large single dose of ethionine (0.6 mmole/animal) the incorporation of radioactivity from putrescine into spermidine was decreased from 490 counts/min per 100 mg (wet wt) of hepatic tissue to 170. The addition of methionine or adenosine triphosphate to liver homogenates obtained from normal rats did not increase spermidine synthesis. A slight increase in spermidine synthesis was evident when adenosine triphosphate was added to homogenates obtained from ethionine-treated rats. This increase however was not very large and the control value was not nearly reached.

In an other experiment (not tabulated) it was observed that an addition of methionine and ethionine alone or together to the incubation medium did not alter the spermidine synthesis in liver homogenates *in vitro*.

In any case these experiments indicate that the formation of S-adenosylethionine and subsequently the lack of S-adenosylmethionine cause an early decrease in the biosynthesis and concentration of spermidine in ethionine-treated rats.

### *Concluding remarks*

All the above data are in agreement with the idea that putrescine also serves as the natural four carbon precursor in polyamine biosynthesis in rat liver. Its concentration is rather low as compared with that of polyamines but this fact can be explained in terms of the rapid turnover rate of putrescine in mammalian tissues. The fate of the radioactivity derived from intraperitoneally administered  $^{14}\text{C}$  putrescine indicated that in all probability the most marked route in the metabolism of exogenous putrescine is its oxidation to carbon dioxide but it is also seemed that a considerable proportion of the putrescine reaching the liver tissue is converted into polyamines at least when compared with other tissues.

There is also indirect evidence that the incorporation of the propylamine moiety from methionine occurs *via* the formation of S-adenosylmethionine. Thus the mechanism for spermidine biosynthesis in rat liver seems to be comparable to that earlier observed in microorganisms.

## II BIOSYNTHESIS OF PUTRESCINE AND POLYAMINES IN REGENERATING RAT LIVER

After the removal of a portion of the mammalian liver a series of morphological and biochemical changes occur in the remaining liver. These changes finally result in the reconstitution of the original tissue mass.

Among the earliest changes taking place after partial hepatectomy are those involving polyamine metabolism. The synthesis of spermidine from  $^{14}\text{C}$  methionine was markedly stimulated as early as 4 to 8 hours after operation being at that time several times higher than that of the sham operated controls (RAINA, JÄNNE and SIIMES 1965, 1966). An increase in the concentration of hepatic spermidine could be observed within the first days after operation while that of spermine decreased slightly during that period. The incorporation of putrescine into spermidine during the early period of regeneration was somewhat peculiar. Like methionine this diamine has been reported to be incorporated more rapidly into spermidine almost immediately after partial hepatectomy (DYKSTRA and HERBST 1965). On the other hand it seemed in our previous study that the incorporation of radioactivity from  $^{14}\text{C}$  putrescine into spermidine even decreased during the first day of regeneration (JÄNNE and RAINA 1966).

The following paragraphs attempt to give more detailed picture of the changes in the biosynthetic pathway of spermidine resulting in an almost two fold increase in the concentration of this polyamine a few days after partial hepatectomy.

### *The contents of putrescine and polyamines in rat liver after partial hepatectomy*

Table 3 lists the changes in the concentrations of putrescine, spermidine and spermine in regenerating rat liver over a period of three days. The animals in this experiment were relatively young and as seen in the control animals the concentration of liver putrescine represented about one tenth of that of spermidine. A marked accumulation of putrescine took place almost immediately after partial hepatectomy. At 4 h after the operation the concentration of putrescine was already more than twice the control value. This high level persisted until 12 h after which a gradual decrease took place. The concentration of this diamine however clearly exceeded the control value even at 72 h after operation. The changes in the

**Table 3** The concentrations of putrescine spermidine and spermine in regenerating rat liver. The animals were one month old female rats five to six animals in each group. Means  $\pm$  standard deviations (SD) are presented. C = unoperated controls. Pu = putrescine Spd = spermidine Sp = spermine

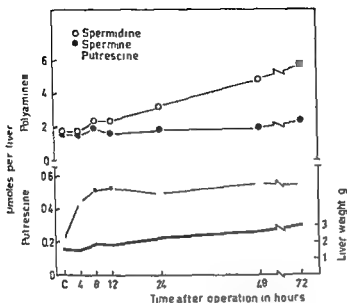
Time after operation (h)	$\mu\text{moles/g}$ (wet wt)			Molar ratios	
	Putrescine	Spermidine	Spermine	Pu/Spd	Spd/Sp
C	133 $\pm$ 28	1 110 $\pm$ 78	973 $\pm$ 53	0.12	1.14
4	294 $\pm$ 38 ***	1 130 $\pm$ 48	976 $\pm$ 56	0.26	1.16
8	272 $\pm$ 21 ***	1 220 $\pm$ 166	997 $\pm$ 41	0.22	1.23
12	282 $\pm$ 26 ***	1 290 $\pm$ 71 **	822 $\pm$ 53 **	0.22	1.57
24	220 $\pm$ 38 **	1 440 $\pm$ 266 *	785 $\pm$ 112 **	0.15	1.84
48	212 $\pm$ 21 ***	1 840 $\pm$ 111	719 $\pm$ 60 ***	0.12	2.55
72	180 $\pm$ 35 *	1 890 $\pm$ 121 ***	772 $\pm$ 67 ***	0.10	2.44

\*  $p < 0.05$  \*\*  $p < 0.01$  \*\*\*  $p < 0.001$ . The significance of the differences as compared with the controls.

concentrations of spermidine and spermine were in agreement with earlier investigations, i.e. an increase in the concentration of spermidine could be seen at 12–24 h after partial hepatectomy while the concentration of spermine at that time was decreased.

The molar ratio of putrescine to spermidine (Pu/Spd) and that of spermidine to spermine (Spd/Sp) are given in the right hand column of Table 3. The ratio of Pu/Spd increased from the initial value of 0.12 to 0.26 at 4 h and was again normalized at 48 h after partial hepatectomy. A more than two-fold increase could also be observed in the ratio of Spd/Sp but this rise occurred later reaching a maximum of 2.55 two days after partial hepatectomy. Some similarities between the regeneration process and physiological aging can be seen with respect to the Spd/Sp ratio. Accordingly the molar ratio of spermidine to spermine was high in young animals but then decreased rapidly with increasing age (JÄNNE, RAINA and SIIMES 1964).

As shown in Fig. 8 the total putrescine content of the residual liver reached a maximum at 4–12 h postoperatively and thereafter remained essentially unchanged up to 3 days. The accumulation of putrescine in the liver was very rapid since the total amount increased from 200  $\mu\text{moles}$



**Fig 8** Total putrescine and polyamine contents in regenerating liver. The animals were the same as in Table 3. The values are expressed as  $\mu\text{moles}$  per residual liver (wet wt). The thick solid line represents the liver weight curve. C = unoperated controls.

to 440 during the first 4 hours. A clear increase in the spermidine content could be seen at 24 h and in general the rise was more gradual than that seen in putrescine. The total spermidine content per residual liver increased over three-fold i.e. from 1700  $\mu\text{moles}$  to 5650 during the period of three days while that of spermine remained practically unchanged during the whole observation period in spite of a marked increase in liver weight (the thick solid line in Fig 8).

#### *Synthesis of putrescine from $^{14}\text{C}$ ornithine in regenerating liver*

What is the origin of the accumulated putrescine after partial hepatectomy? This putrescine might be derived from the intestine and the increased hepatic concentration of putrescine could depend on the decreased detoxication capacity of the regenerating liver. However as shown in Fig 8 it is obvious that the biosynthesis of putrescine from ornithine is greatly

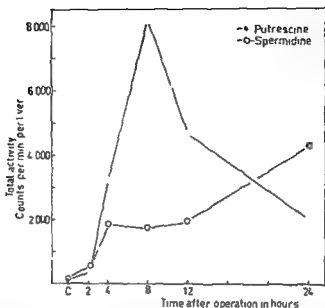


Fig. 1 Incorporation of radioactivity from  $^{14}\text{C}$  ornithine into putrescine and spermidine after partial hepatectomy. 5 month old female rats received  $4\ \mu\text{Ci}$  ( $\approx 0.42\ \mu\text{mole}$ ) of DL ornithine- $5\text{-}^{14}\text{C}$  2 h before analysis. The activities are expressed as counts/min per residual liver (wet wt). Three to four animals in each group. C ~ unoperated controls.

enhanced during the very early period of regeneration. The radioactivity of the putrescine fraction 2 hours after injection with  $^{14}\text{C}$ -ornithine which in the normal liver was 130 counts/min per residual liver thus barely exceeding that of the background increased to about 3000 counts/min at 8 h postoperatively. Thereafter the activity decreased relatively rapidly still exceeding the control value several times at 24 h after partial hepatectomy. The radioactivity from  $^{14}\text{C}$  ornithine was also incorporated into hepatic spermidine and this incorporation increased several fold after partial hepatectomy too. Some interesting features however are seen in the biosynthesis of spermidine from ornithine during early liver regeneration. The increase in the radioactivity of liver spermidine was parallel to that of putrescine only for the first 4 hours after operation; thereafter the radioactivity of the spermidine remained essentially unchanged until 12 h in spite of the considerable increase in putrescine activity at that time. On the other hand at 24 h postoperatively the total spermidine radioactivity derived from  $^{14}\text{C}$ -ornithine clearly exceeded that of putrescine.

*Table 4 Incorporation of radioactivity from  $^{14}\text{C}$  ornithine into liver putrescine and spermidine after partial hepatectomy Specific activities are presented as counts/min per  $\mu\text{mole}$  (ranges in parentheses) See legend for Fig 9 C = unoperated controls*

Time after operation (h)	Specific activity	
	Putrescine	Spermidine
C	1 300 (260—2900)	110 (80—130)
2	2 700 (1100—4000)	370 (310—410)
4	11 100 (8200—14600)	900 (660—1100)
8	16 600 (14800—18900)	1 000 (800—1400)
12	13 900 (9900—17300)	850 (710—870)
24	8 100 (6900—9400)	1 300 (1000—1500)

Table 4 gives the specific activities of putrescine and spermidine obtained from the same experimental animals as in Fig 9. Assuming that the incorporation of radioactivity from  $^{14}\text{C}$  ornithine into hepatic spermidine occurs *via* putrescine one can see that despite a marked increase in the specific activity of the precursor practically no additional activity was incorporated into spermidine between 4 and 12 h after operation. Only at 24 h postoperatively the specific activity of spermidine was clearly increased while that of putrescine had already decreased from its maximum.

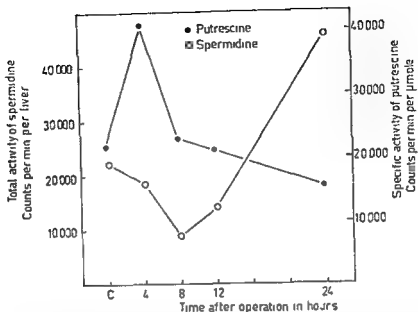
Based on the above data it may be concluded that the accumulation of putrescine in the liver during early regeneration at least partly depends on a considerable enhancement of the biosynthesis of this diamine from ornithine. It also seems evident that the incorporation of radioactivity from  $^{14}\text{C}$  ornithine into spermidine greatly increases during early regeneration and in all probability this incorporation occurs *via* putrescine.

#### *Synthesis of spermidine from $^{14}\text{C}$ putrescine in regenerating liver*

To study the biosynthesis of polyamines from putrescine in more detail a group of rats was subjected to partial hepatectomy and  $^{14}\text{C}$  putrescine was administered as an indicator of the biosynthesis of polyamines.

Fig 10 shows the incorporation of radioactivity from  $^{14}\text{C}$  putrescine into spermidine expressed as counts/min per residual liver and also shows the specific activities of putrescine at appropriate stages of early regeneration.





**Fig 10** Incorporation of radioactivity from  $^{14}\text{C}$  putrescine into spermidine and the putrescine retention in liver after partial hepatectomy Two month old male rats received  $3\mu\text{C}$  ( $=0.3\mu\text{mole}$ ) of putrescine  $1,4^{14}\text{C}$  2 h before analysis The spermidine activities are expressed as count/min per residual liver (wet wt) the putrescine ones as counts/min per  $\mu\text{mole}$  Two to three animals in each group C = unoperated controls

The total spermidine activity decreased as shown in the figure from the control value 22 000 counts/min to 8 100 at 8 h after partial hepatectomy and then again increased being 45 600 at 24 h postoperatively On the other hand except for a peak at 4 h the specific activity of putrescine remained relatively constant during the whole observation period Thus the decreased activity of spermidine after injection of  $^{14}\text{C}$ -putrescine into partially hepatectomized rats can not simply be explained by the isotope diluting effect of markedly increased endogenous putrescine

Table 5 gives the corresponding specific activities of spermidine and spermine The specific activities of spermidine are comparable to those observed in total activities except that the increase shown in total activities at 24 h was not so marked since the liver weight and the concentration of spermidine were already increased at that time

If the simple isotope diluting effect of increased endogenous putrescine can not alone cause the marked reduction in the incorporation of label

**Table 5** Incorporation of radioactivity from  $^{14}\text{C}$  putrescine into liver spermidine and spermine after partial hepatectomy. Specific activities are presented as counts/min per  $\mu\text{mole}$  (ranges in parentheses). See legend for Fig. 10. C = unoperated controls.

Time after operation (h)	Specific activity	
	Spermidine	Spermine
C	8 800 (9000-8650)	360 (360-365)
4	7 000 (6000-8600)	440 (370-580)
8	3 100 (2800-3500)	260 (230-280)
12	4 600 (3700-5450)	300 (280-325)
24	9 200 (6100-11700)	500 (370-590)

from exogenous  $^{14}\text{C}$  putrescine into spermidine during early regeneration there are however other ways of explaining this phenomenon.

The production of putrescine during early regeneration was increased several fold and there can be a preferential use of this endogenous putrescine for spermidine synthesis. Thus the exogenously added  $^{14}\text{C}$ -putrescine should not be equilibrated with the endogenous putrescine pool. This even seems possible since the amounts of  $^{14}\text{C}$  putrescine were negligible as compared with the endogenous hepatic pool of putrescine.

Table 6 presents the specific activities of putrescine and spermidine during early liver regeneration 2 hours after injection with a very large dose (0.25 mmole/animal) of  $^{14}\text{C}$  putrescine. This amount of exogenous

**Table 6** Incorporation of radioactivity from a large dose of  $^{14}\text{C}$  putrescine into liver spermidine and the putrescine retention in liver after partial hepatectomy. Two month old male rats received  $3 \mu$  (= 0.25 mmole) putrescine 2 h before analysis. Three animals in each group. Specific activities are presented as counts/min per  $\mu\text{mole}$  (ranges in parentheses). C = unoperated controls.

Time after operation (h)	Specific activity	
	Putrescine	Spermidine
C	37 600	1 480 (1360-1620)
6	33 800	1 380 (1.40-1440)
8	30 000	1 310 (1250-1370)

putrescine was sufficient to increase the concentration of hepatic putrescine 4 to 5 fold. Interestingly enough the specific activity of spermidine was essentially unchanged at 6 and 8 h after partial hepatectomy as compared with the controls. The specific activity of putrescine also remained relatively constant although a slight dilution of the activity could be seen at 8 h postoperatively. Accordingly it seems obvious that the decreased incorporation of label from exogenous  $^{14}\text{C}$  putrescine as shown in Fig 10 and Table 5 can be explained in terms of the preferential use of endogenous putrescine and the dilution of the exogenous  $^{14}\text{C}$ -putrescine with endogenous putrescine.

*Spermidine synthesis from  $^{14}\text{C}$ -putrescine in liver homogenates obtained from partially hepatectomized rats*

Fig 11 illustrates the synthesis of spermidine from  $^{14}\text{C}$  putrescine in liver homogenates obtained from normal and partially hepatectomized rats.

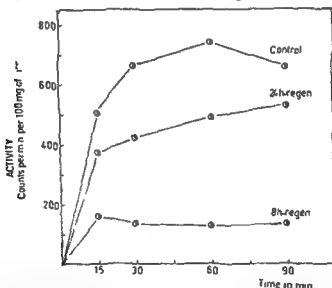


Fig 11 Spermidine synthesis from  $^{14}\text{C}$  putrescine in liver homogenates obtained from normal and partially hepatectomized rats

The animals were two-month old male rats. Control = pooled homogenate obtained from four unoperated animals. 24 h regen = pooled homogenate obtained from 6 rats partially hepatectomized 24 h before incubation. 8 h regen = pooled homogenate obtained from 6 rats partially hepatectomized 8 h before incubation. Each 25 ml flask contained 0.8 g tissue (wet wt) and 0.0285 mM ( $0.15 \mu\text{Ci/ml}$ ) putrescine- $14^{14}\text{C}$  in 250 mM sucrose buffered with 10 mM potassium phosphate buffer at pH 7.8. Other assay conditions as in Fig 5. Each time point represents a mean of three assays.

The differences between the homogenates obtained from unoperated animals and from those subjected to partial hepatectomy 8 or 24 h earlier were in general in agreement with those results observed *in vivo* (Fig 10 and Table 5). The incorporation of label from  $^{14}\text{C}$  putrescine into spermidine was also greatly depressed *in vitro* in liver homogenates at 8 h after partial hepatectomy. The synthesis of spermidine from putrescine *in vivo* seemed to be resumed as early as 24 h after partial hepatectomy and the activities even exceeded those of the control animals at that time. However as shown in Fig 11 under these conditions the incorporation curve of this time point ran clearly under the control curve but was still considerably above that observed in 8 h regenerating liver.

Thus this experiment in addition seems to support the idea that the synthesis of spermidine from putrescine is somewhat inhibited during early liver regeneration. On the other hand the preferential use of endogenous putrescine for spermidine synthesis is not excluded although the liver cells in homogenate are broken and the endogenous and exogenous pools of putrescine could be mixed.

*Incorporation of radioactivity from  $^{14}\text{C}$  arginine into putrescine spermidine and liver total protein after partial hepatectomy*

Arginine can also serve as a precursor of polyamine synthesis in rat liver and the incorporation of label from  $^{14}\text{C}$  arginine into liver spermidine is markedly increased after partial hepatectomy (JÄNNE and RAINA 1966). In microorganisms this amino acid can even act as the main precursor of putrescine synthesis and is converted into putrescine *via* agmatine (MORRIS and PARDEE 1966).

To study the biosynthetic pathway involving the formation of spermidine from arginine in more detail both hepatic putrescine and spermidine were analyzed for their radioactivity after the injection of normal and partially hepatectomized rats with  $^{14}\text{C}$  arginine.

As shown in Fig 12 a marked stimulation of the biosynthesis of putrescine and spermidine from arginine was obvious within a few hours after partial hepatectomy. The total hepatic putrescine activity expressed as counts/min per residual liver increased from about 100 counts/min to 1300 at 8 h postoperatively. Between 8 and 12 h the putrescine activity again rapidly decreased but still clearly exceeded the initial value at -4 h. A similar several fold stimulation was also observed in the incorporation of radioactivity from  $^{14}\text{C}$  arginine into spermidine during liver regeneration.

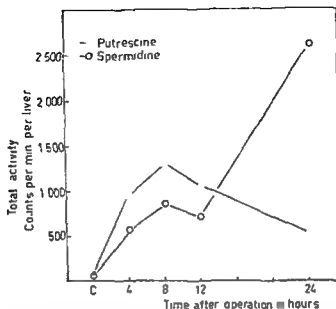


Fig 12 Incorporation of radioactivity from  $^{14}\text{C}$  arginine into putrescine and spermidine after partial hepatectomy 25 month old female rats received 5  $\mu\text{C}$  ( $= 10 \mu\text{mole}$ ) of DL arginine  $5^{14}\text{C}$  3 h before analysis The activities are expressed as counts/min per residual liver (wet wt) Three to four animals in each group C = unoperated controls

The total activity of liver spermidine increased from the control value 50 counts/min to 2600 at 24 h postoperatively In general the shapes of the incorporation curves of both putrescine and spermidine closely resembled those observed after the administration of  $^{14}\text{C}$  ornithine to partially hepatectomized rats

Table 7 represents the corresponding specific activities of hepatic putrescine and spermidine at various stages of the regeneration process The specific activities behaved as did the total activities of putrescine and spermidine

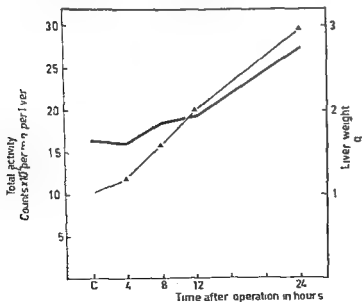
The above results suggested that at least a considerable part of the transfer of label from  $^{14}\text{C}$  arginine into spermidine in rat liver occurs *via* putrescine and thus the diamine putrescine serves as an intermediate in the formation of spermidine from arginine What kind of intermediates and reactions are involved in the biosynthesis of putrescine from arginine is not yet clear The bacterial putrescine is formed directly from ornithine or from arginine *via* agmatine (MORRIS and PARDEE 1965 1966) Both of these mechanisms can also exist in mammalian liver but it is possible that

**Table 7** Incorporation of radioactivity from  $^{14}\text{C}$  arginine into liver putrescine and spermidine after partial hepatectomy. Specific activities are presented as counts/min per  $\mu\text{mole}$  (ranges in parentheses). See legend for Fig. 1. C = unoperated controls

Time after operation (h)	Specific activity	
	Putrescine	Spermidine
C	620 (260—920)	40 (20—60)
4	3000 (2700—3200)	400 (400—430)
8	4700 (3800—5500)	550 (420—700)
12	4100 (2900—5300)	300 (250—510)
24	2200 (1500—2500)	800 (610—1000)

a part of the putrescine formed from arginine is synthesized via ornithine. The above experiments however did not exclude the possibility that agmatine could act as a direct precursor not only for putrescine synthesis but also for spermidine formation in rat liver.

The total amount of the amino acid arginine incorporated into liver



**Fig. 13** Incorporation of radioactivity from  $^{14}\text{C}$  arginine into total liver protein after partial hepatectomy. See legend for Fig. 1. The thick solid line represents the liver weight curve. C = unoperated controls

putrescine and spermidine was very small however as compared with that incorporated into hepatic protein As shown in Fig 13 the incorporation of radioactivity from DL arginine-5  $^{14}\text{C}$  into total liver protein was considerably increased even during the first day after partial hepatectomy The total activity of hepatic protein increased three fold within 24 hours i.e. from 102 000 counts/min per residual liver to 296 000 at 24 h postoperatively On the other hand the total activity incorporated from  $^{14}\text{C}$  arginine into liver putrescine and spermidine was only 3 100 count /min per residual liver at 24 h after partial hepatectomy (Fig 12) i.e. approximately 1 per cent of the activity found at that time in liver total protein This total hepatic protein did not represent all the proteins synthesized in liver tissue since the plasma proteins were not included

Table 8 gives the specific activities of liver protein As shown in the table the most marked increase in the specific activity occurred within the first 12 hours after operation and between 12 and 24 h only an additional increase of 5 per cent could be observed

*Table 8 Incorporation of radioactivity from  $^{14}\text{C}$  arginine into liver total protein after partial hepatectomy Specific activities are presented as counts/min per mg of protein (ranges in parentheses) See legend for Fig 12 C = unoperated controls*

Time after operation (h)	Specific activity
C	320 (270-370)
4	410 (350-460)
8	500 (480-500)
12	600 (520-670)
24	630 (550-670)

*Effect of putrescine concentration on the incorporation of radioactivity from  $^{14}\text{C}$ -methionine into spermidine in liver homogenates*

As reported previously (RAINA JÄNNE and SIIMES 1965 1966) the incorporation of label from  $^{14}\text{C}$  methionine into spermidine was greatly increased during liver regeneration This stimulation of spermidine synthesis from methionine began practically immediately after partial

the incorporation pattern of putrescine was quite different to that of methionine. On the other hand in bacteria and bacterial preparations both these compounds act as natural precursors of spermidine synthesis being incorporated equimolarly into this polyamine. It also seems apparent that the same mechanism is involved in the natural formation of spermidine in animal tissues. In the regenerating liver the synthesis of spermidine from methionine was stimulated several fold immediately after operation contrary to the unchanged or even decreased incorporation of label from  $^{14}\text{C}$  putrescine into spermidine at the same time. Assuming that the formation of spermidine from ornithine and arginine occurred via putrescine the incorporation patterns of these amino acids into spermidine represent the real incorporation of endogenous putrescine into spermidine. Accordingly it must be assumed that the decreased activity of spermidine after the injection of  $^{14}\text{C}$  putrescine into partially hepatectomized rats could depend on the preferential use of endogenous putrescine and the isotope diluting effect of the increased endogenous putrescine.

An indirect attempt was made to also explain the stimulation of spermidine synthesis from  $^{14}\text{C}$  methionine during liver regeneration *in vivo*. The incorporation of radioactivity from  $^{14}\text{C}$  methionine is a very slow reaction *in vivo* and biosynthesis of spermidine from  $^{14}\text{C}$ -methionine *in vitro* without any additions is barely detectable. Fig. 14 presents the incorporation of radioactivity from methionine into spermidine in a liver homogenate obtained from normal rats. Without the addition of unlabelled putrescine the synthesis of spermidine from methionine under these conditions was minimal. With increasing concentrations of putrescine the specific activity of spermidine rose sharply i.e. from 150 counts/min per  $\mu\text{mole}$  without putrescine addition to about 1600 when the medium contained 0.2 mM of DL methionine-2  $^{14}\text{C}$  and 0.2 to 0.3 mM of unlabelled putrescine. A similar increase in spermidine activity could be observed by using 0.1 mM of  $^{14}\text{C}$  methionine and increasing the concentration of unlabelled putrescine. It should be mentioned that the homogenate at zero point of the abscissa also contained their own endogenous putrescine approximately representing a concentration of 0.1 mM. Thus the sharpest rise in the specific activity of spermidine took place when the physiological putrescine concentration in relation to the enzyme was doubled. This experiment could serve as a possible explanation for the enhancement of spermidine synthesis from methionine during liver regeneration. The intracellular putrescine concentration increased just over two-fold within a few hours after partial hepatectomy and thus accumulated putrescine fur



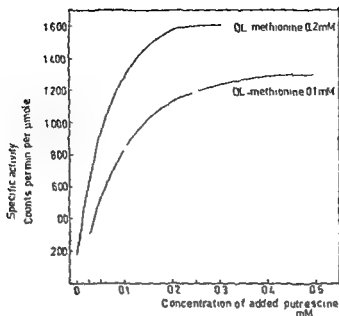


Fig 14 Spermidine synthase from  $^{14}\text{C}$  methionine in liver homogenates obtained from two month old female rats

Each 25-ml flask contained 10 g of tissue (wet wt) 116 mM  $\text{K}^+$  16 mM  $\text{Mg}^{++}$  8 mM  $\text{Na}^+$  124 mM  $\text{Cl}^-$  16 mM  $\text{SO}_4^{--}$  buffered with 10 mM potassium phosphate buffer at pH 7.8 0.1 or 0.2 mM DL-methionine  $2^{14}\text{C}$  (0.5 or 1.0  $\mu\text{C}/\text{ml}$ ) and unlabelled putrescine as indicated. The final volume was 2 ml. The incubation time was 60 min. Other conditions as in Fig 3. Each point represents a mean of three assays.

ther could increase the incorporation of methionine into spermidine. On the other hand the specific activity of spermidine was also increased by raising the concentration of  $^{14}\text{C}$  methionine from 0.1 to 0.2 mM as shown in Fig 14. The concentration of liver free methionine however, has been reported to remain unchanged during regeneration (FERRARI and HARNES 1954). If correct this hypothesis means that the biosynthesis of spermidine from endogenous putrescine is really increased to the same extent as from methionine after partial hepatectomy and the use of exogenous  $^{14}\text{C}$  putrescine differs from that of the endogenous one. Accordingly the incorporation of radioactivity from ornithine or arginine into spermidine could present the actual spermidine synthesis from putrescine and those incorporations were in fact markedly increased after partial hepatectomy.

### Concluding remarks

Fig. 15 summarizes the events involving putrescine and polyamine biosynthesis in regenerating rat liver. In normal livers (Fig. 15 A) the synthesis of putrescine from ornithine is rather slow. On the other hand exogenous putrescine is relatively rapidly converted into spermidine and further into spermine. The concentration of putrescine is low as compared with that of polyamines. At 8 h after partial hepatectomy (Fig. 15 B) the situation is changed. The incorporation of radioactivity from  $^{14}\text{C}$  ornithine into putrescine is increased several fold, the synthesis of spermidine from exogenous putrescine is somewhat inhibited or at least not stimulated and an accumulation of putrescine takes place. At that time the conversion of arginine into putrescine had also reached a maximum. The concentration of spermidine is not essentially changed. At 24 h after operation the situation is again changed (Fig. 15 C). The conversion of ornithine into putrescine is still more rapid than in normal liver but is beginning to decrease. The formation of spermidine from exogenous putrescine is again restored to its initial level or exceeds it. The concentration of putrescine has already decreased from its maximum.

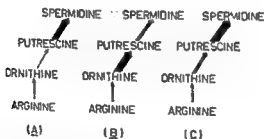


Fig. 15 Spermidine biosynthesis in rat liver after partial hepatectomy  
 (A) Intact liver  
 (B) At 8 h after partial hepatectomy  
 (C) At 24 h after partial hepatectomy

Very little is known of the conversion of arginine into ornithine during liver regeneration but some recent studies have indicated that at least the activity of the enzyme catalyzing this reaction in the mammalian liver remains practically constant during the first day of regeneration. (P. J. L. J. VANNE and G. VANNE)

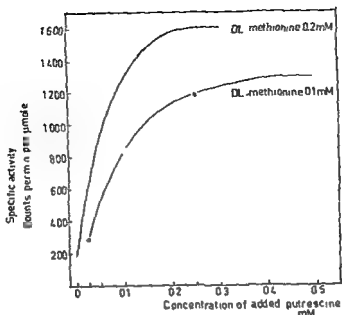


Fig 14 Spermidine synthesis from  $^{14}\text{C}$  methionine in liver homogenates obtained from two month old female rats

Each 25 ml flask contained 10 g of tissue (wet wt) 116 mM  $\text{K}^+$  16 mM  $\text{Mg}^{++}$  8 mM  $\text{Na}^+$  124 mM  $\text{Cl}^-$  16 mM  $\text{SO}_4^{--}$  buffered with 10 mM potassium phosphate buffer at pH 7.8 0.1 or 0.2 mM DL methionine 2  $^{14}\text{C}$  (0.5 or 1.0  $\mu\text{Ci/ml}$ ) and unlabelled putrescine as indicated. The final volume was 2 ml. The incubation time was 60 min. Other conditions as in Fig 5. Each point represents a mean of three assays.

ther could increase the incorporation of methionine into spermidine. On the other hand the specific activity of spermidine was also increased by raising the concentration of  $^{14}\text{C}$  methionine from 0.1 to 0.2 mM as shown in Fig 14. The concentration of liver free methionine however has been reported to remain unchanged during regeneration (FERRARI and HARKNESS 1954). If correct this hypothesis means that the biosynthesis of spermidine from endogenous putrescine is really increased to the same extent as from methionine after partial hepatectomy and the use of exogenous  $^{14}\text{C}$  putrescine differs from that of the endogenous one. Accordingly the incorporation of radioactivity from ornithine or arginine into spermidine could present the actual spermidine synthesis from putrescine and those incorporations were in fact markedly increased after partial hepatectomy.

# *Hepatic putrescine and polyamine concentrations after a single injection of growth hormone into normal rats*

The effect of the growth hormone on the concentrations of putrescine and polyamines is presented in Table 9. The animals in this experiment received a relatively large dose of growth hormone (4 international units, Somacton Ferring) as an intraperitoneal injection and the time course of its action was determined. As shown in the table the concentration of putrescine remained relatively unchanged during the whole period of observation. At 3 to 6 hours after the hormone injection however a slight but not significant increase in putrescine concentration was observed. The changes in the spermidine concentration were not much clearer but a significant increase in the concentration of this polyamine could be observed at 12 h after the hormone injection. On the other hand the concentration of spermine remained quite constant. The molar ratios of these amines are also given in Table 9. Some minor changes can be seen in both ratios Pu/Spd and Spd/Sp during the observation period: i.e. the ratio of Pu/Spd was slightly increased at 3 h and that of Spd/Sp at 12 h after injection. The changes in the concentrations as well as in the molar ratios were not nearly so marked as in the regenerating liver but in any case some similarities could be seen between these two situations.

*Table 9 Concentrations of putrescine, spermidine and spermine in rat liver after growth hormone treatment. On month old male rat received an intraperitoneal injection of 4 international units of growth hormone (Somacton). Means  $\pm$  standard deviations (SD) are presented. Five animals in each group. C = controls treated with solvent only. Pu = putrescine, Spd = spermidine, Sp = spermine.*

Time after injection (h)	$\mu$ moles/g (wet wt)			Molar ratios	
	Putrescine	Spermidine	Spermine	Pu/Spd	Spd/Sp
C	259 $\pm$ 24	1170 $\pm$ 178	1290 $\pm$ 91	0.20	0.91
3	277 $\pm$ 31	1090 $\pm$ 59	1240 $\pm$ 77	0.25	0.88
6	274 $\pm$ 21	1240 $\pm$ 101	1250 $\pm$ 78	0.22	0.99
12	236 $\pm$ 23	1370 $\pm$ 37	1280 $\pm$ 34	0.17	1.07

$p < 0.05$  The significance of the difference as compared with the control group.

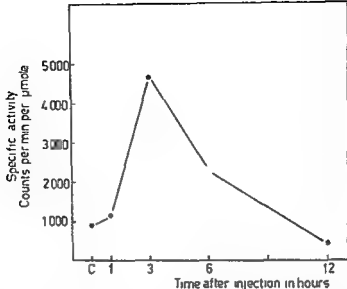


Fig 16 Incorporation of radioactivity from  $^{14}\text{C}$  ornithine into putrescine in rat liver after growth hormone injection One month old male rats were treated with a dose of 4 international units of growth hormone (Somacton)  $3 \mu\text{C}$  ( $= 0.72 \mu\text{mole}$ ) of DL ornithine  $5 \text{ }^{14}\text{C}$  was injected intraperitoneally 1 h before analysis Each point represents a mean of three animals C = controls treated with solvent only

#### *Effect of growth hormone on putrescine synthesis from $^{14}\text{C}$ ornithine*

A slight accumulation of putrescine or spermidine in the livers of growth hormone-treated rats might be due to a retarded degradation of these compounds This possibility was not excluded but as shown in Fig 16 the growth hormone possesses a considerable stimulatory effect on putrescine biosynthesis from  $^{14}\text{C}$ -ornithine There was a rapid rise in the specific activity of putrescine derived from  $^{14}\text{C}$ -ornithine after a single injection of growth hormone The radioactivity of hepatic putrescine increased from its control value of 900 counts/min per  $\mu\text{mole}$  to 4 600 at 3 h after injection with growth hormone and thereafter rapidly decreased to the initial value This stimulation of putrescine synthesis from ornithine seemed to begin practically immediately after the hormone injection and its duration was only a few hours

Although a more than five-fold increase in the biosynthesis of putrescine was obvious only a very slight accumulation, if any of this diamine took place in livers of growth hormone treated rats The reason for this phenomenon should be found in the further metabolism of putrescine

*Biosynthesis of polyamines from  $^{14}\text{C}$  putrescine after growth hormone treatment*

Fig 17 illustrates the biosynthesis of polyamines from  $^{14}\text{C}$  putrescine in growth hormone treated rats. As shown in the figure a single growth hormone injection was followed by a clear increase in the incorporation of label from  $^{14}\text{C}$  putrescine into spermidine. The specific activity of hepatic spermidine increased from the initial value of 1400 counts/min per  $\mu\text{mole}$  to 4600 at 3 h after the growth hormone injection and like that in the formation of putrescine from ornithine this stimulation was also very transitory. The spermidine activity again decreased to the control level at 6 h after the hormone injection. The negligible activity of hepatic spermine remained essentially unchanged during the whole period of observation.

It seems apparent that the pituitary growth hormone also caused early stimulation of spermidine biosynthesis from putrescine in the hepatic tissue.

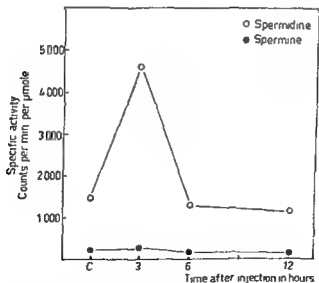


Fig 17 Incorporation of radioactivity from  $^{14}\text{C}$  putrescine into hepatic polyamines after growth hormone treatment. 4 international units of growth hormone (Somacton) was injected into one month old male rats. Analysis 1 h after the injection of 2  $\mu\text{C}$  ( $= 0.21 \mu\text{mole}$ ) of putrescine  $^{14}\text{C}$ . Each point represents a mean of four to five animals. C = controls treated with solvent only.

of normal rats Fig 18 gives more detailed information on the mechanism of this stimulation In this figure it is presented part of the putrescine uptake in the liver after the hormone injection, i.e the radioactivity counted in liver putrescine spermidine and spermine fractions The upper curve in Fig 18 illustrates the total activity found in hepatic putrescine spermidine and spermine 1 h after injection with  $^{14}\text{C}$  putrescine expressed as counts/min per g of liver wet wt The lower curve illustrates the activity counted in the putrescine fraction alone and thus the difference between these two curves represents the polyamines synthesized from  $^{14}\text{C}$ -putrescine As shown in the figure the putrescine activity also increased sharply during the first few hours after the hormone injection A maximum over three times the initial value was observed at 3 h and thereafter a rapid decrease was obvious In addition polyamine biosynthesis increased in the same proportion as this putrescine uptake The solid almost horizontal line in Fig 18 represents the percentage proportion of spermidine activity in the activity counted in the total amine fraction As shown this proportion varied within fairly narrow limits i.e from 20 to 23 per cent during the

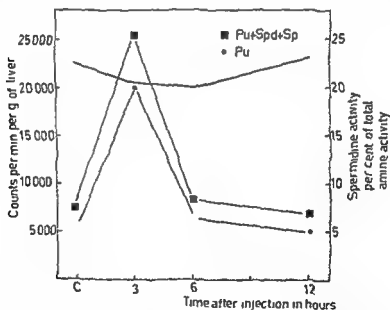


Fig 15 Distribution of radioactivity in liver putrescine and polyamines at 1 h after injection of  $^{14}\text{C}$  putrescine into growth hormone treated rats See legend for Fig 17 The solid almost horizontal line represents the percentage of spermidine activity (count/min per g wet wt) of the total amine activity (count/min per g wet wt) in putrescine spermidine and spermine C = controls treated with solvent only

whole experimental period. Thus it seems that real stimulation of spermidine synthesis from putrescine was not necessarily but the increased activity of spermidine after the injection of  $^{14}\text{C}$  putrescine into growth hormone treated rats could depend on the markedly increased putrescine uptake of the hepatic tissue.

The finding that the growth hormone did not cause real stimulation of spermidine synthesis from putrescine but increased the transport of this diamine into the liver tissue was supported by the discovery indicating that hormone pretreatment did not result in an increase in spermidine synthesis from  $^{14}\text{C}$  putrescine in liver homogenates *in vitro*. Table 10 presents the synthesis of spermidine from  $^{14}\text{C}$  putrescine at 3 h after a single hormone injection. As shown in the table no stimulation of spermidine synthesis could be observed under these conditions. In liver homogenates the transport of the substrate into the tissue or intact cell is eliminated and the result represents solely the biosynthesis of spermidine from putrescine.

In an other experiment (not tabulated) the effect of addition of the growth hormone to the incubation medium was studied. Again no differences between the controls and the growth hormone containing incubations could be observed. It must be realized however that the conditions employed in these experiments might be inadequate for the action of a hormone as compared with an intact tissue or animal.

*Table 10 Effect of growth hormone pretreatment on the incorporation of radioactivity from  $^{14}\text{C}$  putrescine into spermidine in liver homogenates*

The animals were one month old male rats. Three of them received 4 international units of growth hormone (Somacton) and three the solvent of the drug 3 h before incubation. Each .5 ml flask contained 0.8 g of liver tissue (wet wt) and 0.085 mM (= 0.15  $\mu\text{C}/\text{ml}$ ) putrescine-1- $^{14}\text{C}$  in 250 mM sucrose buffered with 10 mM potassium phosphate buffer at pH 7.8. Incubation time was 60 min. Other conditions as in Fig. 5. The values are means  $\pm$  standard deviations (SD) of four assays pooled from three animals.

Pretreatment	Spermidine activity counts/min per 100 mg of tissue
Solvent	4.0 $\pm$ .53
Growth hormone	3.80 $\pm$ .48



*Incorporation of radioactivity from  $^{14}\text{C}$  methionine into liver spermidine spermine and total protein after growth hormone treatment*

Table 12 presents the incorporation of radioactivity from DL methionine  $^{14}\text{C}$  into spermidine spermine and total protein *in vivo* after growth hormone treatment. The experimental conditions in this experiment were the same as previously and the activities of the growth hormone-treated rats represents the situation at 3 h after a single hormone injection. The specific activity of spermidine increased from 350 counts/min per  $\mu\text{mole}$  to 670 i.e. almost two-fold. On the other hand that of spermine merely decreased after the hormone injection and the specific activity of the total hepatic protein counts/min per mg remained essentially unchanged. Based on the above observations it could be assumed that the primary site of growth hormone action in methionine incorporation also depended on the question of the transport. This possibility was not excluded but there were some facts opposing the transport hypothesis. First methionine has been reported to be incorporated roughly equimolarly into both polyamines in rat liver (RAINA 1964). As shown in the table spermine activity remained unchanged after the hormone treatment. If the hormone would only act on the transport of methionine into the liver an increased amount of methionine must also be available for spermine synthesis and the spermine activity is expected to increase in the same way as that of spermidine. Secondly the essentially unchanged activity in the total liver protein also indicated that a specific stimulation of spermidine synthesis was involved.

*Table 12 Incorporation of radioactivity from  $^{14}\text{C}$  methionine into liver polyamines and total protein *in vivo* after growth hormone injection. The animals were one month old male rats receiving 4 international units of growth hormone (Somacton) or solvent of the drug and 3  $\mu\text{C}$  ( $\approx 0.63 \mu\text{mole}$ ) of DL methionine  $^{14}\text{C}$  3 h before analysis. Three animals in each group. Specific activities are presented as count/min per  $\mu\text{mole}$  of polyamines or mg of protein (ranges in parentheses).*

Treatment	Specific activity		
	Spermidine	Spermine	Protein
Solvent	350 (310-390)	310 (250-410)	710 (700-720)
Growth hormone	670 (550-840)	270 (240-280)	730 (690-810)

### *Concluding remarks*

There are some similarities between liver regeneration and growth hormone action on polyamine biosynthesis in rat liver. Both situations cause an increase in the concentration of liver spermidine and the mechanism by which the biosynthetic pathway of this polyamine is stimulated seems to be to some extent comparable. In both cases the formation of putrescine from its precursors was greatly enhanced while the incorporation of radioactivity from exogenous  $^{14}\text{C}$  putrescine into spermidine was not necessarily increased. On the other hand because of the markedly increased formation of putrescine it seemed that the amount of endogenous putrescine being incorporated into spermidine was greater in the livers of partially hepatectomized or growth hormone-treated rats than in the controls which was apparently reflected in the increased incorporation of label from methionine into spermidine.

## Discussion

The results of the present investigation demonstrate the presence of putrescine a common diamine in the bacterial world as also occurring in rat liver tissue. Although the presence of this diamine in the tissues of some mammalian species has been known for years putrescine has not really been included among the normal compounds found in fresh mammalian tissues. The significance of putrescine in mammalian polyamine metabolism is still partly obscure but based on the present results it seems that this diamine serves as the natural precursor of polyamine synthesis also in mammalian tissues. The putrescine concentration in rat liver is only one tenth to one third of spermidine. On the other hand its conversion into polyamines is relatively rapid in mammalian tissues too indicating a rapid turnover rate of this compound as compared with the slower turnover rates of the polyamines spermidine and spermine (SIIMES 1967).

The conversion of putrescine into polyamines in the liver or other tissues is not at all the major metabolic route for intraperitoneally administered putrescine since this compound is very rapidly oxidized into carbon dioxide. The location of this oxidation as well as the possible intermediates are not known.

Apparently a considerable portion of hepatic putrescine is converted into polyamines in an intact rat liver. Spermidine synthesis also occurs *in vitro* in liver slices and homogenates even if this reaction is possibly not as rapid as *in vivo*.

The behaviour of the polyamines (RAINA 1963) and of the diamines (CALDARERA BARBIROLI and MORUZZI 1965) in the developing chick embryo correlate well with the other metabolic alterations occurring during embryonic development. The behaviour of the putrescine concentration during the postnatal growth of the rat is a more complex question. The putrescine concentration is just at its lowest during the first weeks after birth and thereafter rapidly increases. A converse mode of behaviour is shown in the concentration of spermidine it is at its highest at birth and thereafter decreases (JÄNNE, RAINA and SIIMES 1964). The putrescine concentration correlates to some extent with the growth phases of

the rat but this correlation is not very clear. During the first weeks of postpartum life the liver of the rat does not grow as rapidly as the whole body. Thus lag phase in liver growth lasts only one to two weeks after which the liver weight and its percentage of the total body weight rapidly increases (OLIVER, BALLARD, SHIELD and PENTLEY 1964; OLIVER and BLUMER 1964). The period of rapid growth is characterized by an increase in deoxyribonucleic acid concentration followed by an increase in the concentration of ribonucleic acid (OLIVER and BLUMER 1964; WINICK and NOBLE 1965). Thus the putrescine concentration is low during the lag phase and then rapidly increases reaching a maximum between the first and second month. The conversion of putrescine into spermidine also increases at least *in vitro* during the first months of life. And interestingly enough the activity of the S-adenosylmethionine synthetizing enzyme has also been reported as increasing in the mouse and rabbit liver in a similar manner to that of spermidine synthesis from putrescine in the rat liver (HANCOCK 1966).

At first sight the behaviour of the concentration of putrescine and its conversion into spermidine are difficult to compare with the behaviour of the spermidine concentration during the postnatal development of the rat. The biosynthesis of spermidine from putrescine increases while the concentration of this polyamine decreases. Thus it should be assumed that the increased synthesis of spermidine from putrescine prevents a marked decrease in spermidine concentration due to increased formation of spermine from spermidine at that time (Fig. 6A; SIIMES 1967).

The results of the present investigation do not absolutely illustrate the role of putrescine as acting as the main four-carbon precursor in the synthesis of spermidine in rat liver for other precursors have been neither excluded nor shown. The incorporation of radioactivity from  $^{14}\text{C}$  methionine into spermidine and spermine in rat tissues has been demonstrated by RAINA (1964). The idea that these compounds incorporate equimolarly together to form a spermidine molecule was supported by experiments dealing with the early action of ethionine on spermidine synthesis from putrescine. Ethionine, the ethyl analogue of methionine, causes a deprivation of tissue adenosine triphosphate (SHULL 1962; BARTELS and HOHORST 1963) because of the formation of S-adenosylethionine (MODY, BULBA, HOLOWECKY and STEKOL 1963; RAINA, JÄNNE and SIIMES 1964; SHULL, McCONOMY, VOGT, CASTILLO and FARBER 1966). An other early effect of ethionine is a decrease in the hepatic spermidine concentration (RAINA, JÄNNE and SIIMES 1964). The

inhibition of spermidine synthesis from putrescine as shown in this investigation seems to be the result of a decrease in the substrate concentration i.e. S adenosylmethionine. The lower level of this substrate is reflected by a decrease in the incorporation of putrescine into spermidine. The very slight effects obtained by adding methionine or adenosine triphosphate to the incubation medium could depend on the slow formation of S adenosylmethionine from its precursors under these *in vitro* conditions.

Compared with the developmental growth of an animal some extreme experimental conditions provide much more information on the metabolism of polyamines in rat hepatic tissue. Thus the removal of a portion of the mammalian liver provokes a series of biochemical events including changes in both putrescine and polyamine biosynthesis. The morphological as well as biochemical changes taking place in regenerating liver are reviewed in detail elsewhere (HARKNESS 1957, BUCHER 1963). Some of these however will be mentioned here.

The most rapid changes in liver chemical components occur in the area of storage products i.e. there is an early depletion of liver glycogen and accumulation of fats in the liver (HARKNESS 1957). The hepatic concentrations of some amino acids and related compounds, including glutamic and aspartic acids, lysine and ethanolamine phosphoric ester increase rapidly after partial hepatectomy (FERRARI and HARKNESS 1954).

The most interesting and also quite rapid changes occur in the area of nucleic acid metabolism. Almost immediately after partial hepatectomy there is a marked stimulation in the incorporation of labelled precursors into ribonucleic acid (BUCHER 1963, FUJIOKA, KOGA and LIEBERMAN 1963, BRESNICK 1965). The incorporation of  $^{14}\text{C}$ -orotate into hepatic ribonucleic acid begins to arise immediately after operation and reaches a maximum as early as 5 h postoperatively (FUJIOKA, KOGA and LIEBERMAN 1963). On the other hand, deoxyribonucleic acid synthesis shows a rise later, at about 15 to 18 h after partial hepatectomy (BUCHER 1963). The synthesis of ribonucleic acid catalyzed by ribonucleic acid polymerase is stimulated at about 6 h and reaches its maximum at 12 to 24 h after operation (BUSCH, CHAMBERMAN, MANDEL and WEILL 1962, RO and BUSCH 1967).

As shown in the present investigation the accumulation of putrescine takes place almost immediately after partial hepatectomy i.e. it reaches a maximum of about twice the initial concentration as early as 4 h

postoperatively. This accumulation is probably not a result of the diminished detoxication capacity of the remaining liver since a several fold increase in the incorporation of label from  $^{14}\text{C}$ -ornithine into this diamine was observed. The significance of the accumulation of putrescine is still obscure but the increased biosynthesis of putrescine from ornithine could be explained in terms of some other events taking place during the liver regeneration.

Fig 19 illustrates a possible association of putrescine and polyamine metabolism with the urea cycle and the early steps of pyrimidine biosynthesis. The total urea output is somewhat lowered during the first three days after partial hepatectomy (HARKNESS 1957) and there are some changes in the activities of the enzymes concerning urea formation. Only the activity of two enzymes however changes markedly during liver regeneration. There is a decrease in the activity of ornithine transcarbamylase (COHEN and SALLACH 1961 KIM and COHEN 1965) and a smaller decrease in the activity of arginase (RÄIHÄ JÄNNÉ and SUHKONEN 1967). The other enzymes concerned urea biosynthesis in rat liver remain essentially unchanged. The common precursor carbamyl phosphate does not only serve as a substrate for the urea forming system but also acts as a precursor in pyrimidine synthesis. In hepatic tissue one of the first steps of pyrimidine synthesis is catalyzed by aspartate transcarbamylase. In the case of rat liver the observation has been made that aspartate transcarbamylase activity increases considerably during the first days after partial hepatectomy.

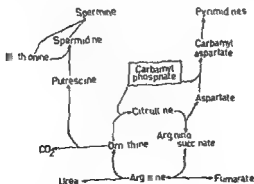


Fig 19 Schematic representation of the postulated interrelation ships between urea, pyrimidine and polyamine biosynthesis in rat liver

(CALVA and COHEN 1959 KIM and COHEN 1965 BRESNICK 1965) Thus it is possible that the amount of carbamyl phosphate available for urea formation decreases during liver regeneration because of a marked increase in aspartate transcarbamylase activity This hypothesis is supported by the finding that carbamyl phosphate synthetase activity remains quite constant during regeneration although there is a greater need for this compound in pyrimidine synthesis (KIM and COHEN 1965)

In the present study it was shown that the incorporation of radio activity from  $^{14}\text{C}$ -ornithine into putrescine increases several fold within a few hours after partial hepatectomy Thus there are two different systems competing for the substrates of ornithine transcarbamylase one utilizing carbamyl phosphate for pyrimidine synthesis and the other using ornithine for the formation of putrescine and polyamines During active regeneration these two competing systems are considerably stimulated and the activity of ornithine transcarbamylase decreases On the other hand the maximum decrease in ornithine transcarbamylase activity occurs at about 4 to 5 days after partial hepatectomy (KIM and COHEN 1965 RÄIHÄ JÄNNE and SUIHKONEN 1967) in contrast with the peaks of aspartate transcarbamylase activity at 48 h and putrescine formation from ornithine at 11 h respectively The following explanation for this apparent discrepancy will be postulated Ornithine transcarbamylase activity might decrease in an adaptive manner because of the abolished or markedly reduced supply of the substrates carbamyl phosphate and ornithine during the first day after partial hepatectomy Assuming that the half life of ornithine transcarbamylase is like that of arginase (SCHIMKE BROWN and SMALLMAN 1963 SCHIMKE 1964) 4 to 5 days a decrease of 50 per cent in the enzyme activity should occur not until 4 to 5 days after the end of the enzyme protein synthesis If correct this hypothesis means that during liver regeneration the substrate pools carbamyl phosphate and ornithine remain essentially unchanged but that the substrates are utilized in a different manner for the urea cycle pyrimidines and polyamines respectively

The behaviour of spermidine biosynthesis from putrescine during early regeneration remains partly unexplained The incorporation of radioactivity from exogenous putrescine into spermidine is in fact decreased or remains unchanged for a few hours after partial hepatectomy On the other hand an increase in the concentration of spermidine can be seen as early as 24 h postoperatively and the synthesis of spermidine from methionine is stimulated practically immediately after operation (RAINA JÄNNE and

SIIMES 1965 1966) The incorporation of label from ornithine and arginine into putrescine is increased several fold after partial hepatectomy and the activity of hepatic spermidine derived from the above mentioned precursors also increased very markedly immediately after operation. The formation of spermidine from ornithine and arginine occurs in all probability in putrescine in rat liver. Thus it must be assumed that the use of exogenously administered putrescine differs from that of endogenous putrescine resulting in an apparent decrease in the incorporation of radioactivity from exogenous  $^{14}\text{C}$  putrescine into spermidine during liver regeneration. The increase in the synthesis of spermidine in regenerating liver as shown in the incorporation pattern of methionine into this polyamine (RAINA, JÄNNE and SIIMES 1965 1966) could be explained in terms of increased substrate concentration of putrescine after partial hepatectomy rather than by a specific enzyme induction at this step. This hypothesis is supported by the fact that also in normal liver homogenates increasing concentrations of putrescine caused a several fold increase in the incorporation of radioactivity from  $^{14}\text{C}$  methionine into spermidine.

The significance of the accumulation of spermidine and putrescine in rat liver during the early stages of regeneration is not at all clear but it could be assumed that they are in some way related to nucleic acids especially ribonucleic acid. Putrescine and polyamines exert stabilizing effects on cellular polyamions in microorganisms (TABOR and TABOR 1964). In animal tissues too there are connections between these compounds and nucleic acids e.g. in regenerating rat liver (DYKSTRA and HERBST 1965, RAINA, JÄNNE and SIIMES 1965 1966) in regard to the subcellular distribution (RAINA and TELARANTA 1967) in developing chick embryos (RAINA 1963, CALDARERA, BARBIROLI and MORUZZI 1964) etc. The simultaneous stimulation of nucleic acid synthesis and that of their stabilizers should be practical for a cell population undergoing rapid growth.

Spermidine, spermine and putrescine stimulate bacterial ribonucleic acid polymerase *in vitro* (KRAKOW 1963, FOX and WEISS 1964). The activity of this enzyme is also increased during the first day of regeneration (BUSCH, CHAMBERMAN, MANDEL and WEILL 1966, RO and BUSCH 1967) and it is not impossible that putrescine and polyamines could affect the polymerase enzyme in a regulatory manner even in animal tissues.

Based on the results of the present investigation it seems that the increase in putrescine and spermidine concentration after partial hepatectomy occurred as the result of prior stimulation of putrescine biosynthesis from



its precursors The changes in polyamine metabolism after growth hormone treatment are at least to some extent comparable with those observed in regenerating liver Among the earliest events following growth hormone treatment are the stimulation of amino acid transport into cells (RIGGS and WALKER 1960) and changes involving ribonucleic acid synthesis (TALWAR PANDA SARIN and TOLANI 1962 KORNER 1963 1964 TALWAR and GUPTA 1964 WIDNELL and TATA 1964 1966 PEGG and KORNER 1965) The growth hormone also stimulates the incorporation of amino acids into hepatic protein in hypophysectomized rats (KORNER 1959) The stimulation of protein synthesis seems to be mediated through primary stimulation of messenger ribonucleic acid synthesis (KORNER 1963)

The growth hormone also causes early stimulation of putrescine synthesis from ornithine The incorporation of label from  $^{14}\text{C}$ -ornithine reached a maximum at 3 h after the hormone injection On the other hand it seems evident that the synthesis of spermidine from putrescine is not necessarily stimulated but that the increased activity of spermidine after the injection of growth hormone treated rats with  $^{14}\text{C}$  putrescine could depend on the markedly increased putrescine uptake by the liver tissue Thus again the net increase in spermidine concentration could be the result of increased formation of endogenous putrescine from ornithine and this in turn could cause the rise in the incorporation of methionine into spermidine

The hormonal stimulation of amino acid transport into tissues as reported by RIGGS and WALKER (1960) is not excluded in the case of ornithine and methionine but the increase in the concentration of liver spermidine and the specific stimulation of spermidine synthesis from methionine as compared with spermine indicate that the effects caused by the growth hormone on polyamine metabolism, can not solely be dependent on the stimulation of the transport of radioactive precursors into the liver tissue

Thus it seems that the same step in polyamine biosynthetic pathway as in regenerating liver is stimulated after growth hormone treatment The time-course of this stimulation is very close to that previously observed in the activity of ribonucleic acid polymerase after growth hormone administration (WIDNELL and TATA 1966) The activity of this enzyme reached a maximum almost twice the initial value at 4 h after a single injection of growth hormone

The present investigation demonstrated three different growth processes developmental and regenerative growths and an anabolic condition caused

by the pituitary growth hormone. These three situations are not directly comparable but some similarities can be seen. In any case the results of the present study make it justifiable to conclude that putrescine and polyamines are in some way related to the growth processes of an animal tissue.

## Summary

The purpose of this study was to investigate the biosynthetic pathway involving putrescine, spermidine and spermine biosynthesis in the rat liver.

The distribution of the polyamines spermidine and spermine in animal tissues is a relatively well documented area but there are only a few studies dealing with the occurrence of the diamine putrescine in mammalian tissues.

Putrescine was shown to occur in rat liver and its concentration varied markedly in relation to age. During the first two weeks of life the concentration of putrescine in the liver was low, under 100  $\mu$ moles per g wet wt, increasing rapidly until the age of six weeks and thereafter again decreasing almost to the initial level. The maximum concentration of liver putrescine was 270  $\mu$ moles/g at the age of six weeks, corresponding to about one third of the spermidine concentration at that time.

Putrescine was rapidly converted *in vivo* into liver polyamines. 4 hours after an intraperitoneal injection of putrescine-1,4- $^{14}$ C the activity in the liver spermidine and spermine fractions represented about 73 per cent of the activity disappeared from the hepatic putrescine fraction. On the other hand the total radioactivity found in liver polyamines represented only a small percentage of that of the added dose.

Intraperitoneally injected  $^{14}$ C putrescine was very rapidly oxidized into carbon dioxide. Thus the radioactivity expired as  $\text{CO}_2$  was 37 per cent of the total dose 2 h after injection with  $^{14}$ C putrescine. Three per cent of the dose was found in the liver polyamines and 2.5 per cent in the polyamines of other tissues.

Spermidine synthesis from putrescine in liver homogenates *in vitro* was also demonstrated. The synthesis of spermidine from putrescine *in vitro* varied in a similar manner to that of putrescine concentration in relation to age.

Administration of DL-ethionine to rats caused a several fold decrease in the incorporation of label from putrescine into spermidine within a few

hours *in vivo* This inhibitory effect of ethionine was only transitory and the spermidine synthesis was almost normalized after 4-day treatment The inhibitory effect of ethionine on the spermidine synthesis in liver homogenates was slightly diminished by adding methionine or adenosine triphosphate to the incubation media

Many changes in putrescine and polyamine synthesis were observed in rat liver undergoing regeneration after partial hepatectomy The concentration of liver putrescine increased over two fold within 4 hours after partial hepatectomy and its biosynthesis from ornithine and arginine was greatly increased immediately after operation reaching a maximum at 8 h postoperatively The label from ornithine and arginine was also incorporated into spermidine whose activity increased several fold after partial hepatectomy too On the other hand the incorporation of radioactivity from exogenous  $^{14}\text{C}$  putrescine into spermidine did not increase during regeneration and even decreased when a carrier free isotope was used A similar decrease in the synthesis of spermidine from putrescine was observed under *in vitro* conditions These results suggest that the increase in spermidine synthesis in regenerating rat liver is due to a prior increase in putrescine biosynthesis and accumulation

Some similarities were observed in putrescine and polyamine metabolism between rats subjected to partial hepatectomy or treated with the pituitary growth hormone The growth hormone caused a slight accumulation of liver spermidine at 12 h after a single hormone injection The concentrations of liver putrescine and spermine remained essentially unchanged On the other hand this hormone possessed a considerable stimulatory effect on putrescine synthesis from  $^{14}\text{C}$ -ornithine since more than 5 fold increase in the incorporation of radioactivity from ornithine into putrescine was observed as early as 3 h after the hormone injection In addition to the synthesis of spermidine from putrescine, the uptake of putrescine possibly also increased after growth hormone treatment The radioactivity from  $^{14}\text{C}$  methionine was incorporated into spermidine almost two times more rapidly in growth hormone-treated rats than in the control animals The specific activity of spermine and total liver protein remained essentially unchanged Hormone pretreatment did not stimulate the incorporation of label from  $^{14}\text{C}$ -putrescine into spermidine in liver homogenates *in vitro*

# References

- AMES B N and D T DUBIN The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid *JbiolChem* 1960 235 769-775
- AMES B N D T DUBIN and S M ROSENTHAL Presence of polyamines in certain bacterial viruses *Science* 1958 127 814-816
- BACHRACH U ■ ABZUG and A BEKIERKUNST Cytotoxic effect of oxidized spermine on Ehrlich ascites cells *Biochim biophys Acta* 1967 134 174-181
- BARTELS H and H J HOHORST Zur Wirkung des Aethionin auf den metabolitstatus der Rattenleber *Biochim biophys Acta* 1963 71 214-216
- BRESNICK E Early changes in pyrimidine biosynthesis after partial hepatectomy *JbiolChem* 1965 240 2550-2556
- BREWER E N and H P RUSCH Control of DNA replication Effect of spermine on DNA polymerase activity in nuclei isolated from *Thysanurus polycc phalum* *Biochem biophys Res Commun* 1966 25 579-584
- BUCHER N L R Regeneration of mammalian liver In *International Review of Cytology* Vol 15 Ed G H Bourne and J F Danielli Acad Press New York 1963 245-300
- BUSCH S P CHAMBER P MANDEL and J D WEILL The effect of partial hepatectomy on the ribonucleic acid polymerase of rat liver *Biochem biophys Res Commun* 1962 7 255-258
- CALDARERA C M B BARBIROLI and G MORUZZI Polyamines and nucleic acids during development of the chick embryo *Biochem J* 1965 97 84-88
- CALDARERA C M C COZZANI and M S MORUZZI Polyamines and nucleic acids in rat liver subjected to ionizing radiation. *Experientia* 1966 22 579-580
- CALVA E and P P COHEN Carbamyl phosphate aspartate transcarbamylase activity in regenerating rat liver *Cancer Res* 1959 19 679-683
- CANTONI G L and J DURELL Activation of methionine for transmethylation. II The methionine activating enzyme Studies on the mechanism of the reaction *JbiolChem* 1957 225 1033-1048
- COHEN P P and H J SALLACH Nitrogen metabolism of amino acids In *Metabolic pathways* Vol 2 Ed D M Greenberg Acad Press New York 1961 1-78
- COHEN S ■ N HOFFNER M JANSEN M MOORE and A RAINA Polyamines RNA synthesis and streptomycin lethality in a relaxed mutant of *E coli* strain 15 TAU *Proc nat Acad Sci (Wash)* 1967 57 721-725
- COHEN S S and J LICHTENSTEIN Polyamines and ribosome structure *Jbiol Chem* 1960 235 2111-2116
- DUBIN D T and S M ROSENTHAL The acetylation of polyamines in *Escherichia coli* *JbiolChem* 1960 235 776-782
- DUDLEY H W and O ROSENHEIM Notes on spermine *Biochem J* 1923 19 1034-1036
- DYKSTRA W G Jr and E J HERBST Spermidine in regenerating liver Relation to rapid synthesis of ribonucleic acid *Science* 1965 149 428-429
- FERRARI V and R ■ HARKNESS Free amino-acids in liver and blood after partial hepatectomy in normal and adrenalectomized rats *JPhysiol* 1954 124 443-463
- FISCHER F G and H BOHN Über die Bestimmung von Spermin Spermidin und anderen biogenen Aminen nach papierelektrophoretischer Abtrennung und ihre Mengenverhältnisse in tierischen Organen. *Hoppe Seyler's Z physiol Chem* 1957 308 108-115

- FOX C F and E B WEISS Enzymatic synthesis of ribonucleic acid II Properties of the deoxyribonucleic acid primed reaction with *Micrococcus luteus* ribonucleic acid polymerase *J Biol Chem* 1964 239 175-185
- FRIEDMAN M E and U BACHRACH Inhibition of protein synthesis by spermine in growing cells of *Staphylococcus aureus* *J Bact* 1966 92 49-55
- FUJII T and R KOYAMA Nucleic acid contents of various organs in rats during postnatal growth *Endocrinol Jap* 1962 9 66-73
- FUJIOKA M M KOGA and I LIEBERMAN Metabolism of ribonucleic acid after partial hepatectomy *J Biol Chem* 1963 238 3401-3406
- GALE E F The production of amines by bacteria I The decarboxylation of amino acids by strains of *Bacterium coli* *Biochem J* 1940 34 392-413
- GOLDSTEIN J The effect of spermine on the accumulation of nucleic acids and protein in mammalian cells *Exp Cell Res* 1965 37 494-497
- GOLDSTEIN J Resistance of RNA to thermal denaturation in the presence of polyamines *Biochim biophys Acta* 1966 113 620-623
- GREENE R C Incorporation of the carbon chain of methionine into spermidine *J Am chem Soc* 1957 79 3929
- GUGGENHEIM M *Die biogenen Amine* 4 Aufl Verlag von S Karger Basel 1951 pp 619
- HAM R G Putrescine and related amines as growth factors for a mammalian cell line *Biochem biophys Res Commun* 1964 14 34-38
- HANCOCK R L S adenosylmethionine synthesizing activity of normal and neoplastic mouse tissues *Cancer Res* 1966 26 2425-2430
- HARKNESS R D Regeneration of liver *Brit med Bull* 1957 13 87-93
- HARRISON G A The approximate determination of spermine in single human organs *Biochem J* 1933 27 1152-1156
- HERBST E J and E E SNELL Putrescine and related compounds as growth factors for *Hemophilus parainfluenzae* 7901 *J Biol Chem* 1949 181 47-54
- HERBST E J R H WEAVER and E L KEISTER The gram reaction and cell composition Diamines and polyamines *Arch Biochem* 1958 75 171-177
- HERSCHKO A S AMOZ and J MAGER Effect of polyamines and divalent metals on *in vitro* incorporation of amino acids into ribonucleoprotein particles *Biochem biophys Res Commun* 1961 5 46-51
- HIGGINS G M and M ANDERSON Experimental pathology of the liver Restoration of the liver in the white rat following partial surgical removal *Arch Path* 1931 12 186-202
- HIRSCH J G The essential participation of an enzyme in the inhibition of growth of tubercle bacilli by spermine *J exp Med* 1953a 97 327-343
- HIRSCH J G Spermine oxidase an amine oxidase with specificity for spermine and spermidine *J exp Med* 1953b 97 345-355
- HAMALAINEN R Über die quantitative Bestimmung des Spermins im Organismus und sein Vorkommen in menschlichen Geweben und Körper flüssigkeiten *Acta Soc Med Duodecim* 1947 Series A 23 97-165
- JÄNNE J Effect of growth hormone on putrescine and polyamine synthesis in rat liver *Scand J Clin Lab Invest* In press
- JÄNNE J and A RAINA Further observations on the biosynthesis of polyamines in regenerating rat liver *Acta chem scand* 1966 20 1174-1176
- JÄNNE J A RAINA and M SIIMES Spermidine and spermine in rat tissues at different ages *Acta physiol scand* 1964 62 352-358
- KIM S and P P COHEN Transcarbamylase activity in fetal liver and in liver of partially hepatectomized parabiotic rats *Arch Biochem* 1965 100 421-428

- KORNER A The effect of hypophysectomy of the rat and of treatment with growth hormone on the incorporation of amino acids into liver proteins in a cell free system *Biochem J* 1959 73 61-71
- KORNER A Growth hormone control of messenger RNA synthesis *Biochem biophys Res Commun* 1963 13 386-389
- KORNER A Regulation of the rate of synthesis of messenger ribonucleic acid by growth hormone *Biochem J* 1964 94 449-456
- KOSTYO J L Changes in polyamine content of rat liver following hypophysectomy and treatment with growth hormone *Biochem biophys Res Commun* 1966 23 150-155
- KRAKOW J S Ribonucleic acid polymerase of *A. otobacter vinelindii* III Effect of polyamines *Biochim biophys Acta* 1963 7 506-571
- LOWRY O H N J ROSEBROUGH A L FARR and W J RANDALL Protein measurement with the Folin phenol reagent *J Biol Chem* 1951 193 265-275
- MARTIN W H Jr M J PEICZAR Jr and P A HANSEN Putrescine as a growth requirement for *Neisseria* *Science* 1952 116 483-484
- MITRA S and P KAESBERG Interaction of polyamines with turnip yellow mosaic virus RNA *Biochem biophys Res Commun* 1963 11 146-151
- MODY U S BULBA O HOLOWECKY and J A STEKOL The availability of the adenine moiety of S adenosyl derivatives of methionine and ethionine for the synthesis of liver AMP ADP ATP and RNA in male and female rats *Fed Proc* 1963 22 471
- MOLLER M L and Y H KIM Effects of putrescine and magnesium on the ribosomes of a *Pseudomonas* *Biochem biophys Res Commun* 1965 20 46-50
- MORRIS D R and A B PARDEE A biosynthetic ornithine decarboxylase in *Escherichia coli* *Biochem biophys Res Commun* 1965 20 607-707
- MORRIS D R and A B PARDEE Multiple pathways of putrescine biosynthesis in *Escherichia coli* *J Biol Chem* 1966 241 3129-3135
- OCHOA M Jr and I B WEINSTEIN Spermine inhibition of polypeptide synthesis in a subcellular system derived from the L 1210 mouse ascites leukemia *Biochim biophys Acta* 1965 95 176-179
- OLIVER I T F J BALLARD J SHIELD and P J BENTLEY Liver growth in the early postpartum rat *Develop Biol* 1962 4 108-116
- OLIVER I T and W F C BLUMER Metabolism of nucleic acids during liver maturation in the neonatal rat *Biochem J* 1964 91 559-565
- PEGG A E and A KORNER Growth hormone action on rat liver RNA polymerase *Nature (Lond)* 1965 205 904-905
- RAINA A Spermine and spermidine in normal human blood *Scand J Clin Lab Invest* 1962a 14 318-319
- RAINA A Biosynthesis of spermine and spermidine in the developing chick embryo *Acta chem scand* 1962b 16 2463-2464
- RAINA A Studies on the determination of spermidine and spermine and their metabolism in the developing chick embryo *Acta physiol scand* 1963 60 Suppl 18 1-81
- RAINA A On the biosynthesis of spermidine and spermine in the rat *Acta chem scand* 1964 18 2189-2189
- RAINA A and S S COHEN Polyamines and RNA synthesis in a polyauxotrophic strain of *E. coli* *Proc Nat Acad Sci (Wash)* 1966 55 1587-1593
- RAINA A J JÄNNE and M. SIMES Effect of ethionine on spermidine spermine and adenine nucleotides in rat liver *Acta chem scand* 1964 18 1804-1806

- RAINA A, J JÄNNE and M SIIMES Polyamines and nucleic acids in regenerating rat liver In *Abstracts of the 2nd Meeting of Federation of European Biochemical Societies* (A 114) Vienna 1965
- RAINA A, J JÄNNE and M SIIMES Stimulation of polyamine synthesis in relation to nucleic acids in regenerating rat liver *Biochim biophys Acta* 1966 123 197-201
- RAINA A and T TELARANTA Association of polyamines and RNA in isolated subcellular particles from rat liver *Biochim biophys Acta* 1967 138 200-203
- RIGGS T R and L M WALKER Growth hormone stimulation of amino acid transport into rat tissues *in vivo* *J biol Chem* 1960 235 3603-3607
- RO T S and H BUSCH Studies on RNA polymerase of nuclear fractions of regenerating liver *Biochim biophys Acta* 1967 134 181-187
- ROSENTHAL S M and C W TABOR The pharmacology of spermine and spermidine Distribution and excretion *J Pharmacol exp Ther* 1956 116 131-138
- RÄIHA N, J JÄNNE and J SUIHKONEN Effect of partial hepatectomy on enzymes concerned with urea synthesis in rat liver *Scand J clin Lab Invest* 1969 19:99
- SCHIMKE R T The importance of both synthesis and degradation in the control of arginase levels in rat liver *J biol Chem* 1961 236 3808-3817
- SCHIMKE R T, M B BROWN and E T SMALLMAN Turnover of rat liver arginase *Ann NY Acad Sci* 1963 102 587-601
- SCHLENK F and J L DAINKO Effects of ribonuclease and spermine on yeast cells *Arch Biochem* 1966 113 127-133
- SHIMIZU H, Y KAKIMOTO and I SANO Spermine and spermidine in blood cells and a possible occurrence of spermine in cell nucleus *Arch Biochem* 1963a 110 368-372
- SHIMIZU H, Y KAKIMOTO and I SANO Changes in concentration of polyamines in the developing mouse brain *Nature (Lond)* 1965b 207 1196-1197
- SHULL K H Hepatic phosphorylase and adenosine triphosphate levels in ethionine treated rats *J biol Chem* 1962 237 1734-1735
- SHULL K H, J MCCONOMY, M VOGT, A CASTILLO and E FARBER On the mechanism of induction of hepatic adenosine triphosphate deficiency by ethionine *J biol Chem* 1966 241 5060-5070
- SIEKEVITZ P and G E PALADE Cytochemical study on the pancreas of the guinea pig VII Effects of spermine on ribosomes *J cell Biol* 1962 13 217-232
- SIIMES M A Incorporation of radioactivity from spermidine- $C^{14}$  into spermine  $C^{14}$  in rat liver In *Abstracts of the 3rd Meeting of Federation of European Biochemical Societies* (F 27) Warsaw 1966
- SIIMES M Studies on the metabolism of  $14^{14}C$  spermidine and  $14^{14}C$  spermine in the rat *Acta physiol scand* 1967 Suppl 298 1-66
- SIIMES M and J JÄNNE Polyamines and their biosynthesis in Ehrlich ascites cells *Acta haematol* 1967 1 815-817
- SPAHR P F Amino acid composition of ribosomes from *Escherichia coli* *J molec Biol* 1962 4 395-406
- STADIE W C and B C RIGGS Microtome for the preparation of tissue slices for metabolic studies of surviving tissues *in vitro* *J biol Chem* 1944 154 647-690

- STEKOL J A Biochemical basis for ethionine effects on tissues In *Advances in enzymology* Vol 25 Ed F F Nord Interscience Publishers New York 1963 369-393
- TABOR C W and P D KELLOG The effect of isolation conditions on the polyamine content of *E coli* ribosomes *Jbiol Chem* 1967 242 1011-1052
- TABOR C W and H TABOR Transport systems for 1,4-diaminobutane spermidine and spermine in *Escherichia coli* *Jbiol Chem* 1966 241 3714-3723
- TABOR H The stabilization of *Bacillus subtilis* transforming principle by spermine *Biochem biophys Res Commun* 1961 4 228-231
- TABOR H S M ROSENTHAL and C W TABOR Biosynthesis of spermidine and spermine from putrescine *Fed Proc* 1956 15 367
- TABOR H S M ROSENTHAL and C W TABOR The role of putrescine and methionine in the enzymatic synthesis of spermidine in *Escherichia coli* *J Am chem Soc* 1957 79 2978-2979
- TABOR H S M ROSENTHAL and C W TABOR The biosynthesis of spermidine and spermine from putrescine and methionine *Jbiol Chem* 1958 233 907-914
- TABOR H and C W TABOR Spermidine spermine and related amines *Pharmacol Rev* 1964 16 245-300
- TALWAR G P S L GUPTA and F GPOS Effect of growth hormone on ribonucleic acid metabolism 3 Nature and characteristics of nuclear subfractions stimulated by hormone treatment *Biochem J* 1961 97 565-572
- TALWAR G P N C PANDA G S SARIN and A J TOLANI Effect of growth hormone on ribonucleic acid metabolism: 1 Incorporation of radio active phosphate into ribonucleic acid fractions of rat liver *Biochem J* 1962 82 173-175
- WEAVER R. H and E J HERBST Metabolism of diamines and polyamines in microorganisms *Jbiol Chem* 1958 233 637-646
- WIDNELL C C and J R. TATA Similarities and differences in the stimulation of nuclear ribonucleic acid synthesis by thyroid hormones growth hormone and testosterone *Biochem J* 1964 93 2 P
- WIDNELL C C and J R. TATA Additive effects of thyroid hormone growth hormone and testosterone on deoxyribonucleic acid-dependent ribonucleic acid polymerase in rat liver nuclei *Biochem J* 1966 98 621-629
- WINICK M and A NOBLE Quantitative changes in DNA RNA and protein during prenatal and postnatal growth in the rat *Develop Biol* 1965 12 451-466
- WREDE F Zur Kenntnis des spermins IV *Hoppe Seyler's Z physiol Chem* 1926 153 291-313
- ZILLIG W W KRONE and M ALBERS Untersuchungen zur Biosynthese der Proteine III Beitrag zur Kenntnis der Zusammensetzung und Struktur der Ribosomen. *Hoppe Seyler's Z physiol Chem* 1959 327 131-143





